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Mammalian Methylmalonate-Semialdehyde Dehydrogenase

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Introduction

Methylmalonate-semialdehyde dehydrogenase (MMSDH), located in the mitochondrial matrix space, catalyzes the irreversible oxidative decarboxylation of malonate semialdehyde and methylmalonate semialdehyde to acetyl-CoA and propionyl-CoA, respectively. These reactions are in the distal portions of the valine and pyrimidine catabolic pathways.^{1,2} MMSDH belongs to the aldehyde dehydrogenase superfamily,^{3,4} but is unique among the members of this family because coenzyme A is required for the reaction and a CoA ester is produced.^{1,2} MMSDH, like other aldehyde dehydrogenases, has esterase activity.⁵ An active site *S*-acyl enzyme is common to both the esterase reaction and the aldehyde dehydrogenase/CoA ester synthetic reaction.⁵ An active site cysteine residue (Cys-285 in rat MMSDH³) is acetylated during hydrolysis of *p*-nitrophenyl acetate by MMSDH. Acetyl-CoA is produced instead of acetate from *p*-nitrophenyl acetate when the reaction is conducted in the presence of coenzyme A.⁵ Long-chain fatty acyl-CoA esters also inactivate MMSDH by acylation of its active site cysteine residue,⁶ presumably by reversal of the above process. Active site acylation has been proposed as a mechanism for the regulation of MMSDH activity *in vivo* by long-chain fatty acids.⁷

Purification of Native Methylmalonate-semialdehyde Dehydrogenase from Rat Liver

Reagents

Buffer A: 20 mM Ammonium acetate (pH 7.5 at 4°), 0.1 mM EDTA, 2 mM dithiothreitol (DTT), 0.5 mM NAD⁺ (grade AA-1)

Buffer B: 25 mM potassium phosphate (pH 7.5), 0.1 mM EDTA, 2 mM DTT, 0.5 mM NAD⁺ (grade AA-1), 10% (v/v) glycerol

Buffer C: 10 mM Tris-HCl (pH 8.0 at 4°), 0.1 mM EDTA, 2 mM DTT, 0.5 mM NAD⁺ (grade AA-1)

Purification Procedure

In this procedure,^{2,8} the enzyme is purified from 300 g of frozen rat liver, previously stored at −70°. Livers are allowed to partially thaw at 4°, and then are homogenized in a Waring blender at the high setting for 1 min in 4 volumes of buffer A supplemented with protease inhibitors (see below, in the section on expression of MMSDH in *E. coli*). The suspension is further homogenized in five portions with a Polytron PT 10 homogenizer (Brinkmann, Westbury, NY) at a setting of 4 for 1 min. The pH is adjusted to 7.5 at 4°, and insoluble material is removed by centrifugation at 100,000g for 60 min at 4°. The clear supernatant is carefully decanted, the pH is adjusted to 6.5 at 4° with acetic acid, and the extract mixed with 600 ml of CM-Sepharose equilibrated with buffer A, pH 6.5, at 4°. The slurry is stirred gently for 30 min and then unbound material (containing the MMSDH) is removed by filtration. The CM-Sepharose is washed twice with 1 volume of buffer A. Filtrates are combined, adjusted to pH 7.0 at 4° with NH₄OH, and mixed with 800 ml of DEAE-Sepharose equilibrated with buffer A, pH 7.0 at 4°. The slurry is mixed for 30 min and unbound material containing MMSDH is removed by filtration. The DEAE-Sepharose is washed three times with buffer A. All washes

are combined, and the pH adjusted to 7.5 at 4° with NH₄OH. This extract is applied at a flow rate of 60–80 ml/hr to a hydroxylapatite column (2.5 × 20 cm) equilibrated with buffer B. MMSDH is eluted with a linear gradient of potassium phosphate (total volume, 500 ml) from 100 to 300 mM prepared in buffer B. The enzyme solution is concentrated to a volume of 10–20 ml under N₂ pressure with a YM10 membrane (Amicon, Danvers, MA) and applied at a flow rate of 50 ml/hr to a Sephacryl S-300 column (2.5 × 95 cm) equilibrated with buffer A (pH 7.5 at 4°). Fractions containing MMSDH are pooled, the pH is adjusted to 6.0 at 4° with acetic acid, and the extract is applied to an S-Sepharose Fast Flow column (1.5 × 10 cm) equilibrated with buffer A (pH 6.0) with 10% (v/v) glycerol. In the presence of NAD⁺, MMSDH does not bind to S-Sepharose and elutes in the void volume, whereas most other proteins remain bound. The purified enzyme is concentrated on a phenyl-Sepharose column dialyzed against buffer C, divided into small aliquots, and stored at –70°. Ten milligrams of the enzyme protein can be purified from 100 g of rat liver with a specific activity of 7–9 units/mg of protein measured with malonate semialdehyde as substrate. One unit is 1 μmol/min at 30°.

Apoenzyme Preparation

To prepare enzyme⁵ depleted of NAD⁺, MMSDH at a concentration of 1–2 mg/ml in buffer C is supplemented with (NH₄)₂SO₄ to 2 M, incubated for 15 min at room temperature, and applied at a flow rate of 1 ml/hr to a Sephadex G-25 Fast Flow column equilibrated with 15 mM potassium phosphate (pH 7.8), 0.1 mM EDTA, and 1 mM DTT. To measure the residual amount of NAD⁺ in MMSDH, 1 mg of enzyme is precipitated with 6% (w/v, final concentration) perchloric acid, the extract is neutralized with potassium hydroxide, and NAD⁺ is measured by an enzymatic end-point assay.⁴ Usually, less than 0.05 mol of NAD⁺ per mole of enzyme is detected.

Activity Assay

Preparation of Malonate Semialdehyde and Methylmalonate Semialdehyde

The ethyl ester diethyl acetal of methylmalonate semialdehyde is synthesized as described by Kupiecki and Coon.⁹ Hydrolysis is carried out at 50° for 4 hr with H₂SO₄. The product is then cautiously neutralized on ice with 6 N KOH, brought to pH 6.4 with 1 M KH₂CO₃, cold-filtered through Whatman (Clifton, NJ) No. 1 filter paper, and stored in small aliquots at –70°. Racemic ethylmalonate semialdehyde is prepared by an identical procedure, starting from the corresponding ethyl ester diethyl acetal (ethylhydroacrylic acid). The ethyl ester diethyl acetal of malonate semialdehyde (ethyl 3,3-diethyloxypionate; Aldrich, Milwaukee, WI) is hydrolyzed in a similar manner except that saponification is completed at room temperature for 2 hr. The neutralized, filtered product is used immediately.

Procedure

Enzyme activity is routinely measured by following the reduction of NAD⁺ at 340 nm with a cocktail consisting of 30 mM sodium pyrophosphate, pH 8.0, adjusted with HCl at room temperature, 2 mM DTT, 2 mM NAD⁺, 0.5 mM CoA, and 0.5 mM malonate semialdehyde or methylmalonate semialdehyde. Reactions are initiated with enzyme. Enzyme activity can also be measured by a coupled assay based on the generation of methylmalonate semialdehyde from L-3-hydroxyisobutyrate by 3-hydroxyisobutyrate dehydrogenase.² MMSDH also hydrolyzes *p*-nitrophenyl acetate. Esterase activity of the enzyme is determined in 50 mM potassium phosphate (pH 7.8) and 0.1 mM EDTA at 30°.⁵ *p*-Nitrophenyl acetate, prepared in acetone to minimize spontaneous hydrolysis, is added to a final concentration of 0.25 mM to initiate the reaction. Acetone does not affect enzyme activity provided its concentration is less than 2% in the assay solution. Esterase activity is followed by *p*-nitrophenol production at 400 nm ($E = 16 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$).

Cloning Strategy Used to Obtain Methylmalonate-semialdehyde Dehydrogenase cDNA

Partial Amino Acid Sequence of Methylmalonate-semialdehyde Dehydrogenase

In this procedure,³ N-terminal protein sequencing is performed with an Applied Biosystems (Foster City, CA) model 477A Pulse Liquid protein sequencer, and the phenylthiohydantoin derivatives are analyzed by reversed-phase high-performance liquid chromatography with a model 120A analyzer. To obtain additional sequence information, MMSDH at a final concentration of 50 $\mu\text{g/ml}$ is digested with lysyl endopeptidase C in 10 mM potassium phosphate (pH 7.5), 0.1 mM EDTA, and 0.1 mM DTT for 60 min at 30° (MMSDH-to-protease ratio, 300:1). A 50-kDa proteolytic fragment produced by this treatment is separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), blotted onto a polyvinylidene membrane, and subjected to amino acid sequence analysis.

Oligonucleotide Probes

The peptide sequences available (SSSSVPTVKLFINGKFVQ and NMNLYSYRLPLGVCAGIATFNFPAG for the N terminus of MMSDH and the 50-kDa proteolytic fragment, respectively) did not allow the design of an oligonucleotide probe with low degeneracy and high melting temperature.³ Degeneracy is reduced, therefore, by incorporating inosine residues in the design of two oligonucleotides based on the peptide sequences underlined above.

Cloning

Degenerate, inosine-containing oligonucleotides are synthesized on the basis of peptide sequence and used to amplify rat liver cDNA by polymerase chain reaction (PCR).³ The first strand of cDNA is synthesized with Maloney murine leukemia virus (Mo-MuLV) reverse transcriptase, using random hexamers according to the manufacturer instructions (Perkin-Elmer Cetus, Norwalk, CT). Thirty-five cycles of PCR are performed with primers A(A/G)CTITT(T/C)ATIGA(T/C)GGIAA(A/G)TT(C/T)GTIGA and GCNGG(G/A)AA(G/A)TT(G/A)AAIGGIGCIAT, using 1 min at 42° for annealing, 3 min at 72° for extension, and 1 min at 94° for denaturation. The PCR product is gel purified, subcloned into M13mpl8 (Bethesda Research Laboratories, Gaithersburg, MD) and sequenced with Sequenase version 2.0 according to suggested procedures (U.S. Biochemical, Cleveland, OH). Nondegenerate oligonucleotides GGACCTTATTCCTACCGCCTGCCTCTGGGGGTG and GGAATCCAAAAGTGACAAATGGATTGACATCCAC are synthesized on the basis of the sequence of PCR product and used for the library screening. Oligonucleotide probes are labeled with [γ -³²P]ATP, using T4 kinase. These probes are used to screen 1.5×10^6 individual plaque-forming units (PFU) from rat liver λ gt11 cDNA library (Clontech Laboratories, Palo Alto, CA) essentially as described by Sambrook *et al.*¹⁰ Hybridization conditions are as follows: 6× SSC (150 mM sodium chloride, 15 mM sodium citrate, pH 7.5), 5× Denhardt's solution [0.1% (w/v) bovine serum albumin, 0.1% (w/v) polyvinylpyrrolidone, 0.1% (w/v) Ficoll 400], 0.1% (w/v) SDS, heat-denatured salmon sperm DNA (0.1 mg/ml), and the radiolabeled probe (2×10^6 cpm/ml) at 55° for 17 hr. The filters are washed with 6× SSC–0.1% (w/v) SDS four times at room temperature, and once with 2× SSC–0.1% (w/v) SDS at 55° for 5 min and autoradiographed. Positive plaques are purified through four cycles of screening.

Procedure Used to Obtain 5' Coding Region of Methylmalonatesemialdehyde Dehydrogenase cDNA

In this procedure,³ a specific primer is designed to hybridize to bases 145–177 of the coding strand of the rat cDNA: oligo I (GAAAGAAGATGCTGGATACCATGTGGAGTITAC). External primers (one for each insert orientation) are synthesized to correspond to bases 4266–

4288 (GGTGGCGACGACTCCGGAGCCCG) and bases 4323–4352 (TTGACACCAGACCAACTGGTAATGGTAGCG) of the *Escherichia coli* lactose operon flanking the *EcoRI* insertion site in λ gt11 (primers L and R, respectively). Phage DNA from 1 ml of amplified cDNA library (Clontech Laboratories) stock (titer, 10^{10} PFU/ml) is purified by conventional techniques¹⁰ and used as a template for PCR. Each reaction mixture consists of specific primer, one external primer, and 300–500 ng of purified phage DNA along with deoxynucleoside triphosphates, buffer, and *Taq* polymerase, as per the manufacturer instructions (GeneAmp; Perkin-Elmer Cetus). Template is denatured at 94° for 1 min, primers are annealed at 55° for 1 min, and chains are polymerized at 72° for 2 min for 35 cycles with a 7-min extension at 72° added to the final cycle. A PCR product is found to hybridize specifically to a 27-bp internal oligonucleotide corresponding to bases 118–144 (TTTAGAAGAAACCTGCAGGATCCGGGC) of the rat cDNA. The band is subcloned and sequenced.

Expression of Wild-Type Methylmalonate-semialdehyde Dehydrogenase in *Escherichia coli*

A full-length cDNA encoding MMSDH is constructed from two partial cDNA clones. The 700-bp cDNA and the 1400-bp cDNA corresponding to the 5' and 3' ends of the mRNA, respectively, are digested with *KpnI* and *AatII* restriction endonucleases. The fragments are purified by agarose gel electrophoresis and ligated using T4 DNA ligase. The resulting construct is 2 kb, which includes coding sequence for 10 amino acids of the 32-residue leader peptide. The leader peptide and the 3'-noncoding sequence are subsequently removed by PCR amplification with primers flanking the mature coding region. The cDNA encoding mature polypeptide is ligated into the pGEX-2T expression vector and cotransfected into TG-1 *Escherichia coli* cells with the expression plasmid pGroESL (obtained as a kind gift from A. Gatenby, Du Pont, Wilmington, DE). To facilitate purification, the expression vector encodes an MMSDH–glutathione *S*-transferase fusion protein (MMSDH–GST) that is cleavable with thrombin, pGroESL carries the cDNAs for chaperonins GroES and GroEL. Although MMSDH is detected in *E. coli* in the absence of chaperonins by Western blot analysis, the resulting protein is insoluble (Fig. 1B, lane 2), and was inactive. Coexpression with chaperonins has a dramatic effect on the recovery of soluble, active MMSDH activity (Fig. 1A, lane 4). Thus, coexpression of GroEL and GroES is now routinely used in the preparation of recombinant MMSDH. TG-1 cells transfected with pGEX-2T-MMSDH and pGroESL are grown in TY medium supplemented with ampicillin (200 μ g/ml) and chloramphenicol (100 μ g/ml) to an OD₆₀₀ of 0.5–0.7. Induction is initiated by adding isopropyl- β -D-thiogalactopyranoside (IPTG) to 0.2 mM. Cells are harvested by centrifugation and suspended in phosphate-buffered saline (PBS) containing 0.1% (v/v) 2-mercaptoethanol and protease inhibitors phenylmethylsulfonyl fluoride (50 μ g/ml), leupeptin (50 μ g/ml), and benzamidine (5 mM). Cells are sonicated and centrifuged to remove the insoluble fraction. The supernatant is applied to glutathione-agarose under gravity flow. The agarose column is washed with PBS until protein cannot be detected in the flowthrough (<1 μ g/ml) by the Bradford assay. The fusion protein is then eluted with 10 mM glutathione in 50 mM Tris-HCl (pH 8.0)–1 mM DTT. Free glutathione is removed by dialysis against thrombin cleavage buffer [50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 2.5 mM CaCl₂, 0.1% (v/v) 2-mercaptoethanol]. Optimal conditions required to fully cleave the fusion protein with thrombin are determined experimentally (Fig. 2). Four milligrams of MMSDH–GST is incubated with 6 μ g of thrombin at room temperature. Aliquots of the reaction mix are taken at the indicated times and the reaction is stopped by boiling in SDS–PAGE loading buffer. Complete cleavage is achieved after 15 min of incubation with thrombin (Fig. 2). MMSDH is separated from glutathione transferase by a Mono Q column (Pharmacia, Piscataway, NJ) with a linear salt gradient [0–500 mM NaCl in 10 mM Tris-HCl (pH 8.5), 1 mM DTT]. Fractions containing active MMSDH are combined

and concentrated with Amicon concentrators (molecular mass cutoff, 30 kDa). Approximately 1.3 mg of active recombinant protein with a specific activity of 0.14 U/mg as measured by a coupled assay is obtained from 1 liter of culture (Fig. 3).

Preparation and Expression of Methylmalonate - semialdehyde Dehydrogenase Mutants

Cys-285 in MMSDH³ corresponds to Cys-243 in rat cytosolic class 3 aldehyde dehydrogenase,⁴ which is conserved among all aldehyde dehydrogenases⁴ and is part of the active site. To test the role of the corresponding cysteine residue in MMSDH, Cys-285 has been replaced in MMSDH with alanine by site-directed mutagenesis. Glu-268, conserved among all aldehyde dehydrogenases⁴ except MMSDH, has been suggested to act as a base to deprotonate the active site cysteine residue.¹¹ Asn-251 in MMSDH, which aligns with Glu-209 in rat cytosolic class 3 aldehyde dehydrogenase,⁴ has been replaced with glutamate by site-directed mutagenesis. Mutagenesis is performed on MMSDH cDNA cloned into single-stranded M13 vector with a Sculptor Mutagenesis kit according to the manufacturer instructions (Amersham, Arlington Heights, IL). The mutations are confirmed by sequencing and the resulting mutant cDNAs are cloned into pGEX-2T expression vector. Expression vectors for wild-type MMSDH, cysteine-to-alanine, and asparagine-to-glutamate mutants are cotransfected with pGroESL into TG-1 cells. Only wild-type MMSDH is active. Both mutant proteins are expressed in large amounts in the soluble fraction but both are inactive. Thus, Cys-285 is essential for MMSDH function, and substitution of asparagine in position 251 with negatively charged glutamate (as in aldehyde dehydrogenase) also inactivates the enzyme, probably by preventing the binding of a negatively charged substrate. Whereas Glu-268 in aldehyde dehydrogenase may participate in the catalytic hydrolysis of the intermediate acyl enzyme,¹¹ in the case of MMSDH this step involves CoA and the formation of propionyl-CoA without hydrolysis.

Characteristics of Native Methylmalonate-semialdehyde Dehydrogenase cDNA Encoding Rat Methylmalonate-semialdehyde Dehydrogenase, and Wild-Type Recombinant Methylmalonate-semialdehyde Dehydrogenase

The monomer molecular mass of the native enzyme, determined by SDS-PAGE, is 58 kDa.² The rat cDNA encodes a mature protein of 503 amino acids, to give a molecular mass of 55,330 Da.³ The native molecular mass, estimated by gel filtration, is 250 kDa, suggesting a tetrameric structure.² The cDNA also encodes a 32-amino acid leader with characteristics expected of a mitochondrial targeting sequence.³ Kinetic constants for the various substrates indicate that both malonate and methylmalonate semialdehydes are physiological substrates.² The purified rat liver enzyme exhibits K_m and V_{max} values of 4.5 μM and 9.4 units/mg of protein with malonate semialdehyde and 5.3 μM and 2.5 units/mg protein with methylmalonate semialdehyde. The pH optimum with methylmalonate semialdehyde is about pH 8, which is appropriate for the mitochondrial matrix space.² MMSDH appears to use both stereoisomers of methylmalonate semialdehyde, but the substrate may racemize spontaneously. Nevertheless, a single semialdehyde dehydrogenase contributes to the oxidation of valine [(S)- isomer], thymine [(R)-isomer], and several compounds catabolized via β -alanine (transaminates to malonate semialdehyde).² Either no activity or low activity is found with the following compounds: acetaldehyde, butyraldehyde, isobutyraldehyde, α -methylbutyraldehyde, and malondialdehyde.² Low but nevertheless detectable activity is found with succinate semialdehyde. It is likewise found that ethylmalonate semialdehyde, an intermediate in alioisoleucine metabolism, is a poor substrate, giving rates less than 10 milliunits/mg protein.² That the enzyme uses this compound poorly is surprising, because an organic academia has been described^{12,13} that is consistent with a defect in a single semialdehyde dehydrogenase responsible for the oxidation of malonate, methylmalonate, and ethylmalonate semialdehydes.

On the other hand, evidence has been presented for an isolated deficiency in MMSDH in a child with developmental delay, mild methylmalonic aciduria without any increase in malonate, ethylmalonate, or β -alanine.¹⁴ MMSDH is quite sensitive to inhibition by NADH (K_i of 3.1 μ M, competitive with NAD⁺).² Thus, an increase in the mitochondrial NADH- to-NAD⁺ ratio in response to fatty acid oxidation may inhibit MMSDH and perhaps divert methylmalonate semialdehyde to β -aminoisobutyrate by transamination.

MMSDH possesses the catalytic ability to hydrolyze *p*-nitrophenyl acetate with concomitant formation of an *S*-acyl enzyme.⁵ Deacylation of the *S*-acyl enzyme intermediate is the rate-limiting step in the esterase reaction, and the activity is regulated by NAD⁺, NADH, and CoA, suggesting that coenzyme binding in the active site may induce conformational changes in the enzyme that affects the accessibility of an enzyme-thioester intermediate for deacylation.⁵ Limited proteolysis studies carried out with lysyl endopeptidase C, chymotrypsin, and trypsin demonstrated NAD⁺ protection against proteolysis in both the N-terminal and C-terminal parts of the intact enzyme.⁸ Indeed, our initial efforts to purify the enzyme from rat liver were frustrated until we discovered the protection afforded by NAD⁺.² On the other hand, *S*-acylation of the enzyme with *p*-nitrophenyl acetate prevents the stabilizing conformational change induced by NAD⁺.⁸ These findings may have physiological significance because they suggest that acylation may make the enzyme more susceptible to proteolysis. MMSDH is also subject to *S*-acylation at its active site cysteine residue by long-chain fatty acyl-CoA esters.⁶ Acylation by long-chain fatty acids has been proposed to be a physiologically important mechanism for the regulation of MMSDH activity.^{6,7}

Site-directed mutagenesis studies have revealed that Cys-285 is essential for MMSDH function, and that substitution of asparagine in position 251 with negatively charged glutamate (corresponds to Glu-268 in aldehyde dehydrogenase) inactivates the enzyme, probably by preventing the binding of a negatively charged substrate. Whereas Glu-268 in aldehyde dehydrogenase may participate in the catalytic hydrolysis of an intermediate *S*-acyl enzyme, in the case of MMSDH this step involves CoA and the formation of propionyl-CoA without hydrolysis.

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Fig. 1.

Expression of MMSDH-GST fusion protein in the presence and absence of GroEL and GroES "chaperonin" proteins. Lanes 1–3, expression of MMSDH in the absence of GroEL and GroES; lanes 4–6, expression of MMSDH in the presence of GroEL and GroES; lanes 1 and 4, homogenates after sonication; lanes 2 and 5, pellet of insoluble proteins after centrifugation; lanes 3 and 6, soluble fraction. (A) SDS-PAGE analysis; (B) corresponding Western blot analysis of MMSDH expression.

Fig. 2. SDS-PAGE analysis of MMSDH-GST cleavage with thrombin. Samples of 30 μ g were taken at the indicated time points (0, 1, 5, 10, 15, and 20 min) from a mixture of 4 mg of MMSDH-GST and 6 μ g of human thrombin (Sigma) incubated at room temperature, and the reaction was stopped by boiling in SDS-PAGE gel loading buffer.

Fig. 3. Purification of recombinant MMSDH. Lane 1, cell homogenate; lane 2, soluble protein fraction; lane 3, eluate from affinity glutathione-agarose column; lane 4, cleavage of fusion protein with thrombin; lane 5, purified recombinant MMSDH after separation from GST on a Mono Q column; lane 6, molecular weight standards.