

## NOTES

### (R)-Citramalate Synthase in Methanogenic Archaea

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**The *Methanococcus jannaschii* gene MJ1392 was cloned, and its protein product was hyperexpressed in *Escherichia coli*. The resulting protein was purified and shown to catalyze the condensation of pyruvate and acetyl coenzyme A, with the formation of (R)-citramalate. Thus, this gene (*cimA*) encodes an (R)-citramalate synthase (CimA). This is the first identification of this enzyme, which is likely involved in the biosynthesis of isoleucine.**

Citramalate (2-methylmalate) is a biochemical intermediate known to be involved in several aspects of bacterial metabolism, including, among others, the anaerobic metabolism of glutamate via the methylaspartate pathway of *Clostridium tetanomorphum* (2). In this pathway, glutamate is converted via L-threo- $\beta$ -methylaspartate [(2S,3R)-3-methylaspartate] to mesaconate [(E)-2-methyl-2-butenedionic acid], which is then hydrated by citramalate hydrolyase (15, 16) to L-(+)-citramalate (S-citramalate). The resulting citramalate is then cleaved by citramalate lyase to pyruvate and acetate (1). Hydration of citraconic acid [(Z)-2-methyl-2-butenedionic acid] to D-(-)-citramalate (R-citramalate) has also been described (13, 18). A similar pathway for the metabolism of itaconate, via itaconyl coenzyme A (CoA) and citramalyl-CoA, to acetyl-CoA and pyruvate has also been reported (5, 6). The formation of either D-(-) or L-(+)-citramalate by the direct condensation of acetyl-CoA and pyruvate appears never to have been directly observed, but both of these reactions have been proposed as a key step in a number of biosynthetic pathways. Among these is the formation of isoleucine via the pyruvate pathway, which, based on  $^{13}\text{C}$  nuclear magnetic resonance labeling studies (7–9, 15), is the major pathway for isoleucine formation in many species of methanogenic archaea. Labeling studies in other organisms have produced similar results (4, 23).

As part of studies aimed at establishing the identity of genes in the methanogens that were possibly involved in the biosynthesis of coenzymes, we cloned and overexpressed the *Methanococcus jannaschii* gene MJ1392 and identified the reaction catalyzed by the encoded enzyme. We report here that this gene product is (R)-citramalate synthase. This is the first report of the identification of such an enzyme, which we now designate CimA, which is the product of the *cimA* gene.

**Identification, cloning, and high-level expression of the gene product.** The approach that was used to identify this gene was the outcome of other work aimed at identifying the reactions catalyzed by archaeal enzymes with sequences homologous to homocitrate synthase (NifV) (24). The searches for these

genes in the genomes of the methanogens *M. jannaschii* (3) and *Methanobacterium thermoautotrophicum*  $\Delta\text{H}$  (16) were performed with the blast\_to\_rpraze method (The Institute for Genomic Research, Rockville, Md.). The genome of *M. jannaschii* was found to contain three genes homologous to the homocitrate synthase gene (*nifV*) (MJ0503, MJ1392, and MJ1543), and the *M. thermoautotrophicum*  $\Delta\text{H}$  genome had three genes homologous to the homocitrate synthase gene (*nifV*) (MTH1630, MTH723, and MTH1481). The protein products from one or more of these genes were targets for the enzymes involved in the biosynthesis of  $\alpha$ -ketosuberate (10) and thus coenzyme B (7-mercaptoheptanoylthreonine phosphate). Since we did not know the reactions catalyzed by the protein products of each of these genes, we cloned each gene to determine the reactions involved.

Plasmid construct AMJGS60, containing the *M. jannaschii* gene MJ1392, and the PUC18 vector were obtained from The Institute of Genomic Research/American Type Culture Collection microbial genome special collection. The oligonucleotide primers used to direct the PCR formation of a specific gene cartridge had the following sequences: 5' CATGCATA TGATGGTAAGGATATTTGAT 3' and 5' GATCGGATCC TTAATTCAATAACATATTGAT 3'.

The high-level expression of the MJ1392 gene product in the *Escherichia coli* host strain BL21(DE3) was accomplished by constructing a gene cartridge in vitro and cloning this cartridge into the pT<sub>7-7</sub> plasmid (20) such that the gene expression is controlled by the T7 phage transcriptional and translational regulatory elements, which in turn are regulated by the *lac* control elements. The recombinant plasmid was transformed into the host strain BL21(DE3) *E. coli* cells, and the BL21(DE3) *E. coli* cells containing the pT<sub>7-7</sub> plasmid, with insert, were grown in LB medium supplemented with 100 mg of ampicillin per ml at 30°C to an absorbance at 600 nm of 1.0. Protein production was then induced by the addition of isopropyl- $\beta$ -D-thiogalactopyranoside (IPTG) to a final concentration of 1 mM. After the addition of IPTG, the cells were cultured for another 2 h, harvested by centrifugation, and frozen at –20°C until used.

**Preparation of cell extract and purification of citramalate synthase.** The activity of the hyperexpressed *M. jannaschii* enzyme was measured in *E. coli* cell extracts prepared by sonication of the *E. coli* cell pellets (100 to 200 mg [wet weight]) in 2 ml of 0.1 M TES [N-tris(hydroxymethyl)methyl-2-aminoeth-

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TABLE 1. Purification of citramalate synthase

Purification step	Vol (ml)	Total amt of protein (mg)	Total activity (nmol/min)	Sp act (nmol/min/mg)	Yield (%)	Fold purification
Crude extract	1	7.6	818	108	100	1.0
45–55% AS <sup>a</sup> precipitate	1	2.3	204	89	25	0.8
Superose 6 fraction <sup>b</sup>	0.5	0.008	23	2,870	2.8	26.5

<sup>a</sup> Ammonium sulfate saturation.

<sup>b</sup> 200  $\mu$ l heated at 60°C for 10 min before separation.

anesulfonic acid], pH 7.5, buffer. High expression of the desired protein was confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (12% polyacrylamide) of the SDS-soluble cellular proteins, which showed a band at the expected molecular mass of 50 kDa, in agreement with the known gene sequence. The amino-terminal sequence of MJ1392 was calculated to be MMVRIFDTTL on the basis of the DNA sequence. The sequence of the overexpressed protein band was found to be MLVRIFDTTL. The difference observed in the second amino acid in these sequences would result from a single base pair substitution in the third base of the methionine codon and could be the result of an error in the gene sequence reported. The similarity between these sequences further supports the likelihood that the desired protein was obtained.

SDS-PAGE analysis of the proteins in both the soluble extract and the resulting insoluble pellet showed that most of the desired protein was in the pellet. Attempts to recover active enzyme from the insoluble protein in the pellet by the methods of Sambrook et al. (14) were not successful. The enzyme was thus purified from the soluble fraction. Induction of protein synthesis in the presence of 1 mM 2-mercaptoethanol or by growth and induction protein synthesis at 30°C did not allow greater solubilization of the desired protein. The protein band or enzymatic activity was not present in the *E. coli* cells that did not contain the gene insert.

Protein purification was initiated by the addition of solid ammonium sulfate (to 45% saturation) to the soluble protein fraction. The sample was then centrifuged at 16,000  $\times$  *g* for 2 min; the resulting supernatant was then brought to 55% ammonium sulfate saturation and centrifuged for 5 min at 16,000  $\times$  *g*. At this point, CimA was located in the pellet, which was then dissolved in buffer containing 20 mM TES buffer (pH 7.0), 5 mM MgCl<sub>2</sub>, and 150 mM KCl.

The resuspended ammonium sulfate sample was then heated at 60°C for 10 min and centrifuged at 16,000  $\times$  *g* for 4 min, and the supernatant was placed on a Pharmacia Superose 6 column equilibrated in the same buffer at a flow rate of 0.5 ml/min. The elution time of the enzyme corresponded to a molecular mass of 93 to 128 kDa, indicating that its functional conformation is that of a dimer. This has been observed for other enzymes analogous to the *nifV* gene product (24). Activity assays, discussed below, were used to monitor the purification steps (Table 1).

The thermostability of the enzyme was measured by incubating samples (100 to 200  $\mu$ l) at various temperatures for 10 min. The denatured protein was removed by centrifugation, and the supernatant was assayed for enzymatic activity as discussed below. The enzyme showed moderate stability to elevated temperatures (Fig. 1) but did not exhibit the stability expected when compared with other thermophilic proteins expressed by gene cloning (19). Increasing the ionic strength with potassium chloride did not alter the thermostability. Other

factors must be present for this protein to maintain its activity at the growth temperatures of the thermophilic *M. jannaschii*.

**Enzymatic activity and substrate specificity.** The enzyme activity was assayed by monitoring the production of CoA over time by a procedure similar to that used by Srere (17). Samples to be assayed were brought to a final volume of 100  $\mu$ l by mixing with TES buffer (0.1 M, pH 7.5), and made 1 mM acetyl-CoA and 1 mM pyruvate by the addition of 0.1 M solutions of these substrates. The resulting solutions were then incubated at 50°C for 1 h. To the resulting incubation mixture were added 50  $\mu$ l of 10 mM 5,5'-dithio-bis(2-nitrobenzoic acid) in 0.1 Tris-HCl (pH 8.0), 70  $\mu$ l of 1 M Tris-HCl (pH 8.0), and distilled water to a total volume of 0.9 ml. The absorbance at 412 nm was recorded and blanked against an identical incubation sample without the pyruvate. The micromoles of HS-CoA produced were calculated from a standard curve generated with known concentrations (0 to 110  $\mu$ M) of 2-mercaptoethanol. The production of CoA was found to be linear over the 1-h time period of the assay, and product formation was a linear function of the amount of enzyme added. The protein concentration of each of the samples was determined by the bicinchoninic acid method (Pierce Scientific Co.), as described by the manufacturer, with bovine serum albumin serving as the standard.

The specific activity of CimA was 2.9  $\mu$ mol/min/mg of protein, which agrees with that observed for other, similar, enzymes (24). The *K<sub>m</sub>*s for pyruvate and for acetyl-CoA have been determined to be 0.85 and 0.14 mM, respectively. The enzyme was specific for acetyl-CoA and pyruvate. Incubation of the enzyme with  $\alpha$ -ketoglutarate,  $\alpha$ -keto adipate,  $\alpha$ -ketopimelate,  $\alpha$ -ketoisovalerate, and acetyl-CoA (each at 2.8 mM) produced no detectable amount of possible condensation

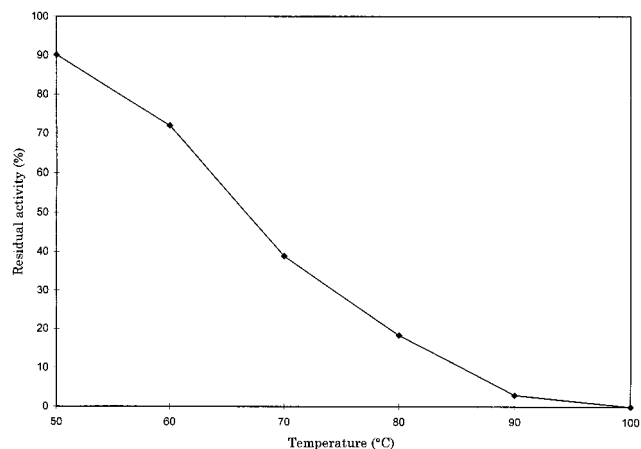


FIG. 1. Residual enzymatic activity after heat treatment at different temperatures for 10 min.

products, as assayed by gas chromatography-mass spectrometry (GC-MS) of the methyl ester derivatives of the possible products (10). Likewise, incubation of the enzyme with acetyl-CoA (1 mM) and propionyl-CoA (0.9 mM) failed to produce 2,3-dimethylmalate. The finding that  $\alpha$ -ketoisovalerate was not a substrate allows this enzyme to be distinguished from 2-isopropylmalate synthase, which is also able to catalyze the condensation of acetyl-CoA and pyruvate (11) to form an isomer of citramalate, but with a  $K_m$  2 orders of magnitude larger than that of  $\alpha$ -ketoisovalerate. Thus, on the basis of the substrates used by this enzyme, the earlier putative identification of the MJ1392 gene product as isopropylmalate synthase was incorrect.

**Analysis of products.** The citramalate product was confirmed by GC-MS of its dimethyl ester derivative, as previously described (10). The product was found to be (*R*)-citramalate by GC-MS with a type G-TA Chiraldex column (0.25 mm by 40 m; Advanced Separation Technologies Inc., Whippany, N.J.) programmed from 95°C to 180°C at 3°C per min. On the Chiraldex GC column, dimethyl (*S*)-citramalate was found to elute before dimethyl (*R*)-citramalate.

The determination that this enzyme is a citramalate synthase further extends the number of natural products that are known to be derived from acetyl-CoA and  $\alpha$ -keto acids.

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#### REFERENCES

- Barker, H. A. 1967. Citramalate lyase of *Clostridium tetanomorphum*. Arch. Mikrobiol. **59**:4–12.
- Buckel, W., and H. A. Barker. 1974. Two pathways of glutamate fermentation by anaerobic bacteria. J. Bacteriol. **117**:1248–1260.
- Bult, C. J., O. White, G. J. Olsen, L. Zhou, R. D. Fleischmann, G. G. Sutton, J. A. Blake, L. M. FitzGerald, R. A. Clayton, J. D. Gocayne, A. R. Kerlavage, B. A. Dougherty, J.-F. Tomb, M. D. Adams, C. I. Reich, R. Overbeek, E. F. Kirkness, K. G. Weinstock, J. M. Merrick, A. Glodek, J. L. Scott, N. S. M. Geoghagen, J. F. Weidman, J. L. Fuhrmann, D. Nguyen, T. R. Utterback, J. M. Kelley, J. D. Peterson, P. W. Sadow, M. C. Hanna, M. D. Cotton, K. M. Roberts, M. A. Hurst, B. P. Kaine, M. Borodovsky, H.-P. Klenk, C. M. Fraser, H. O. Smith, C. R. Woese, and J. C. Venter. 1997. Complete genome sequence of the methanogenic archaeon, *Methanococcus jannaschii*. Science **273**:1058–1073.
- Charon, N. W., R. C. Johnson, and D. Peterson. 1974. Amino acid biosynthesis in the spirochete *Leptospira*: evidence for a novel pathway of isoleucine biosynthesis. J. Bacteriol. **117**:203–211.
- Cooper, R. A., and H. L. Kornberg. 1964. The utilization of itaconate by *Pseudomonas* sp. Biochem. J. **91**:82–91.
- Cooper, R. A., K. Itiaba, and H. L. Kornberg. 1965. The utilization of aconate and itaconate by *Micrococcus* sp. Biochem. J. **94**:25–31.
- Eikmanns, B., R. Jaenchen, and R. K. Thauer. 1983. Unusual pathway of isoleucine biosynthesis in *Methanobacterium thermoautotrophicum*. Arch. Microbiol. **136**:106–110.
- Ekiel, I., G. D. Sprot, and G. B. Patel. 1985. Acetate and CO<sub>2</sub> assimilation by *Methanotherx concilii*. J. Bacteriol. **162**:905–908.
- Ekiel, I., I. C. P. Smith, and G. D. Sprot. 1984. Biosynthesis of isoleucine in methanogenic bacteria: a <sup>13</sup>C NMR study. Biochemistry **23**:1683–1687.
- Howell, D. M., K. Harich, H. Xu, and R. H. White. 1998. The  $\alpha$ -keto acid chain elongation reactions involved in the biosynthesis of coenzyme B (7-mercaptoheptanoylthreonine phosphate) in methanogenic Archaea. Biochemistry **37**:10108–10117.
- Kohlhaw, G. B., and T. R. Leary. 1970.  $\alpha$ -Isopropylmalate synthase (*Salmonella typhimurium*). Methods Enzymol. **17a**:771–777.
- Oda, Y., S. Suzuki, and H. Katsuki. 1987. Physiological roles of two enzymes with fumarase activity in two pseudomonads. Biochem. Int. **14**:871–878.
- Raghavendra Rao, M. R., S. S. Subramanian, H. I. Rahatekar, and S. V. Paranjape. 1963. Enzymatic hydration of citraconate to (–)citramalate. Biochem. Biophys. Res. Commun. **12**:78–82.
- Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
- Schafer, S., T. Paame, R. Vilu, and G. Fuchs. 1989. <sup>13</sup>C-NMR study of acetate assimilation in *Thermoproteus neutrophilus*. Eur. J. Biochem. **186**:695–700.
- Smith, D. R., L. A. Doucette-Stamm, C. Deloughery, H. Lee, J. Dubois, T. Aldredge, R. Bashirzadeh, D. Blakely, R. Cook, K. Gilbert, D. Harrison, L. Hoang, P. Keagle, W. Lum, B. Pothier, D. Qiu, R. Spadafora, R. Vicaire, Y. Wang, J. Wierzbowski, R. Gibson, N. Jiwani, A. Caruso, D. Bush, H. Safer, D. Patwell, S. Prabhakar, S. McDougall, G. Shimer, A. Goyal, S. Pietrokowski, G. M. Church, C. J. Daniels, J. Mao, P. Rice, J. Nöling, and J. N. Reeve. 1997. The complete genome sequence of *Methanobacterium thermoautotrophicum*  $\Delta$ H: functional analysis and comparative genomics. J. Bacteriol. **179**:7135–7155.
- Srere, P. A. 1969. Citrate synthase. Methods Enzymol. **13**:3–11.
- Subramanian, S. S., and M. R. Raghavendra Rao. 1968. Purification and properties of citraconase. J. Biol. Chem. **243**:2367–2372.
- Suzuki, T., Y. Inoki, A. Yamagishi, T. Iwasaki, T. Wakagi, and T. Oshima. 1997. Molecular and phylogenetic characterization of isopropylmalate dehydrogenase of a thermoacidophilic archaeon, *Sulfolobus* sp. strain 7. J. Bacteriol. **179**:1174–1179.
- Tabor, S., and C. C. Richardson. 1985. A bacteriophage T7 RNA polymerase/promoter system for controlled exclusive expression of specific genes. Proc. Natl. Acad. Sci. USA **82**:1074–1078.
- Tanaka, K., K. Nakamura, and E. Mikami. 1991. Fermentation of *S*-citramalate, citrate, mesaconate, and pyruvate by a gram-negative strictly anaerobic non-spore-former, *Formivibrio citricus* gen. nov., sp. nov. Arch. Microbiol. **155**:491–495.
- Wang, C. C., and H. A. Barker. 1969. Activation of *l*-citramalate hydrolyase from *Clostridium tetanomorphum*. J. Biol. Chem. **244**:2527–2538.
- Westfall, H. N., J. W. Charon, and D. E. Peterson. 1983. Multiple pathways for isoleucine biosynthesis in the spirochete *Leptospira*. J. Bacteriol. **154**:846–853.
- Zheng, L., R. H. White, and D. R. Dean. 1997. Purification of *Azotobacter vinelandii* *nifV*-encoded homocitrate synthase. J. Bacteriol. **197**:5963–5966.