Nucleotide Sequence of the Malate Dehydrogenase Gene of *Thermus flavus* and Its Mutation Directing an Increase in Enzyme Activity*

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The nucleotide sequence of the malate dehydrogenase (mdh) gene from a thermophilic bacterium, *Thermus flavus*, was determined. The amino acid sequence of the *Thermus* malate dehydrogenase resembled that of the porcine heart cytoplasmic enzyme to a certain extent, and Asp-159 and His-167 were identified as possible essential residues for the catalytic function. The mutated mdh gene was also cloned from a spontaneous mutant of *T. flavus* containing a higher activity of the enzyme. Its mutation point was determined to be a single nucleotide exchange from C to T which caused Thr-190 to be substituted by isoleucine. The mutated enzyme showed resistance to substrate inhibition, an increase in both acid optimum pH for the enzyme reaction, and a shift toward a more acid optimum pH for the enzyme reaction.

The Gram-negative bacteria of the genus *Thermus*, which are able to grow at temperatures exceeding 70 °C, are useful sources of highly heat-stable enzymes. The malate dehydrogenase stable at 90 °C has been purified from *Thermus flavus* AT-62, and its enzymatic properties have been analyzed (1, 2). It is a dimeric enzyme composed of two identical subunits, each of M, 35,000. Kinetic analyses of the reversible denaturation-renaturation processes of the enzyme have revealed a strong interaction between the subunits which may play a significant role in the stability of the molecule (3). In addition, an essential histidyl residue in the catalytic site of the enzyme shows an abnormally high pKₐ value at room temperature, which compensates for the decrease in the pKₐ value and the optimum pH of the enzyme which occur at elevated temperatures (4). In order to elucidate these characteristic features of the enzyme, determination of the primary amino acid sequence was required.

We have previously cloned the malate dehydrogenase (mdh) gene of *T. flavus* AT-62 in Escherichia coli (5). In this paper we report the nucleotide sequence of the cloned gene, together with an account of the cloning and sequencing of the mutated mdh gene of *T. flavus*, which encodes a form of the enzyme possessing several altered functions. Comparison of the two sequences reveals a single amino acid exchange causing profound modification of the catalytic function of the enzyme.

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number(s) J020598.

MATERIALS AND METHODS

**Bacterial Strains and Plasmids**—E. coli K-12 5313-5 (ade, thy, mdh, HsdR) (5) was used as the host. A plasmid pTA101 carrying the native mdh gene of *T. flavus* AT-62 (ATCC33923) in the 3-kilobase (kb) HindIII fragment on pBR322 (5) was used for the subcloning and sequencing of the gene. Isolation of the mutant strain will be described below.

**Isolation of a Mutant Strain of *T. flavus* AT-62**—Cells were treated with N-methyl-N'-nitroso-N-nitrosoguanidine and allowed to form colonies. The colonies were replicated to Toyo filter papers and then lysed on the filters. They were sprayed with the following solution: 0.2 M Tris-HCl, pH 8.0, 10 mM malate, 0.08 mM NAD, 15 units of diaphorase, and 0.4 mM nitroblue tetrazolium (6). A colony which turned blue rapidly was picked. The strain *T. flavus* F428 showed malate dehydrogenase 3-fold higher than the parental strain.

**Enzymes and Chemicals**—Restriction enzymes, T4 DNA ligase, T4 polynucleotide kinase, bacterial alkaline phosphatase, and DNA polymerase I (Klenow fragment) were purchased from New England Biolabs Inc. and Takara Shuzo Co. Ltd. HindIII linker was obtained from Takara Shuzo Co. Ltd. pYEJ001 and diaphorase were from Pharmacia P-L Biochemicals. A nick-translation kit and kits for cloning and sequencing by the M13 dideoxy method with [α-32P]ATP were obtained from Amersham International. [γ-32P]ATP for sequencing by the method of Maxam and Gilbert was obtained from New England Nuclear. Deoxy-ITP, NAD, and NADH were from Sigma. Oxalacetic acid and L-malate were obtained from Tokyo Kasei and Wako Pure Chemical Industries, respectively. Blue Sepharose CL-6B was obtained from Pharmacia.

**Cloning of the Mutated mdh Gene**—Chromosomal DNA of *T. flavus* F428 prepared by the method of Seito and Miura (7) was digested with HindIII, and the DNA fragments about 3 kb in size separated by agarose gel electrophoresis were recovered by solubilization of gel with NaClO₃ (8). The recovered fragments were ligated with HindIII-digested pBR322 and introduced into *E. coli* 5313-5. The ampicillin-resistant and tetracycline-sensitive transformants were screened by colony hybridization (9) using the nick-translated HindIII fragment from pTA101 containing the whole native mdh gene. Recombinant plasmid pTM1 carrying the mutated mdh gene in the 3-kb insert at the HindIII site of pBR322 was recovered from the transformant which showed positive hybridization with the probe.

**Subcloning of the Native and Mutated mdh Genes**—pTA101 was partially digested with XhoI or completely digested with Ncol, and the resulting linear DNA was religated to produce pTM1, pTM4, and pTM5. In order to express the native mdh gene effectively, a large part of the insert in pTM1 was subcloned downstream of the synthetic consensus promoter on pYEJ001 as follows. The insert of pTM1 was isolated by HindIII digestion, cleaved with HinfI, and the resulting 1.5-kb HindIII-HinfI fragment was introduced in place of the HindIII fragment containing the Cm' gene in pYEJ001 by using HindIII linker (pTM7). A plasmid, pTM7, giving effective expression of the mutated mdh gene was then constructed as follows. The insert of pTM1 was first digested with BstEII and then partially digested with XhoI to give the 1.1-kb BstEII-XhoI fragment. pTM7 was digested with BstEI and SalI, and the resulting linear DNA containing a small part of the native mdh gene with a major part (2.7-kb HindIII-SalI) of pYEJ001 was ligated with the BstEII-XhoI fragment. Since the mutation point was located in the coding sequence down...
stream at the BstEII site, the mutated gene was expressed efficiently in its construction under the control of the synthetic promoter. Plasmids carrying the chimeric mdh genes composed of the native and the mutated genes, pTM’3, pTM’4, pTM’5, and pTM’6, were constructed by using unique BglII and SacI sites in the coding sequence. The recombinant plasmids were introduced into E. coli 5131-5 to test their ability for directing the synthesis of malate dehydrogenase.

DNA Sequencing—DNA sequencing was performed by the method of Maxam and Gilbert (10) and the dideoxy chain termination method in order to minimize compression of the sequence ladders during polyacrylamide gel electrophoresis due to the high G+C content of Thermus DNA (13).

Assay of Malate Dehydrogenase Activity—Malate dehydrogenase activity was assayed by following the decrease in absorbance of NADH at 340 nm with a Hitachi model 200-10 spectrophotometer at 30 °C. The standard assay mixture (3 ml) contained 33 mM potassium phosphate buffer, pH 7.0, 0.17 mM oxalacetate, 0.15 mM NADH, and an appropriate amount of the enzyme. One unit of the enzyme was defined as the amount catalyzing oxidation of 1 μmol of NADH/min.

Preparation of Crude Cell-free Extracts for Enzyme Assay—E. coli 5131-5 harboring the recombinant plasmids was cultured aerobically to full growth in 10 ml of L-broth containing 50 μg/ml ampicillin in a 10-ml test tube at 37 °C and then transferred to 100 ml of the same medium in a 500-ml shaking flask. After cultivation for 4 h at 37 °C with shaking, the cells were harvested by centrifugation and suspended in 3 ml of 100 mM potassium phosphate buffer, pH 7.0. The cell pellets were then disrupted by sonication, and the sonicate was centrifuged at 10,000 × g for 30 min. The supernatant was heated at 80 °C for 30 min, centrifuged to remove precipitated proteins, and used for the enzyme assay.

Purification of Malate Dehydrogenase—E. coli 5131-5 strain harboring pTM’7 or pTM’7’ was grown aerobically in 3 liters of L-broth containing 50 μg/ml ampicillin at 37 °C. 15 ml of wet cells was disrupted by grinding with alumina and extracted with 30 ml of 10 mM Tris-HCl buffer, pH 7.5. After centrifugation at 10,000 × g for 30 min, the extract was dialyzed twice against 1 liter of the same buffer. The dialysate was heated at 80 °C for 30 min, and the precipitates were removed by centrifugation. The supernatant was applied to a column (2 × 15 cm) of Blue Sepharose CL-6B, and the enzyme was eluted with a linear concentration gradient of NADH (0-300 mM). The eluted active fraction showed a single protein band of the malate dehydrogenase monomer upon SDS-polyacrylamide gel electrophoresis. The yields of the enzymes were 43 and 31% from strain 5131-5 carrying pTM’7 and pTM’7’, respectively.

Determination of the Amino Acid Sequence of Malate Dehydrogenase—Five nanomoles of the purified enzyme was dissolved in 300 μl of 10% SDS by heating at 100 °C for 15 min and applied directly to a Beckman 890D protein/peptide Sequencer. Phenylthiohydantoin derivatives of amino acids were analyzed using a Beckman model 420 high-performance liquid chromatography apparatus. Furthermore, 15 nmol of the enzyme was treated with 9 mg of CNBr in 225 μl of 70% formic acid at 25 °C for 2 days. The resulting peptides were separated by high-performance liquid chromatography, and one of more than 10 peaks was sequenced.

RESULTS

Subcloning and Sequencing of the mdh Gene—The originally cloned mdh gene of T. flavus AT-62 in the 3-kb HindIII fragment in pTA101 was expressed by readthrough from the anti-SS promoter of pBR322 (5). In order to reduce the size of the cloned fragment, we constructed derivative plasmids carrying different parts of the HindIII fragment using pBR322 or pYJE001 as the vector. The enzyme activities directed by these derivatives in E. coli 5131-5 were measured (Fig. 1). The results indicated that the mdh gene is present in a 1250-base pair (bp) HindIII-Xhol restriction fragment.

The nucleotide sequence of the 1.8-kb BamHI-Xhol fragment of pTA101 was determined according to the strategy shown in Fig. 2. The sequence was found to contain an open reading frame composed of 981 bp starting from GTG (nucleotides 620-622) as the initiation codon (Fig. 3). In fact, as described below, it was confirmed that the reading frame corresponded to the malate dehydrogenase, as determined by Edman degradation. The molecular weight of the protein encoded by this coding sequence was calculated to be 35,400, which is almost identical with that of the enzyme subunit (M, 35,000) measured by SDS-polyacrylamide gel electrophoresis (2). In order to confirm that the reading frame actually corresponds to the malate dehydrogenase, the partial amino acid sequences of the NH2-terminal peptide and a peptide obtained by CNBr cleavage were determined by Edman degradation. The identical sequences were found in the amino acid sequence deduced from the DNA sequencing (Fig. 3). The amino acid composition of the enzyme calculated from the nucleotide sequence was in good agreement with that obtained by chemical analysis of the purified enzyme (2) (Table I).

A typical Shine-Dalgarno sequence (14) was present 6 bp upstream from the initiation codon, but no consensus promoter sequences found in other prokaryotes (15) were found in the upstream region. Two inverted repeat structures (ΔG = -42.0 (nucleotides 1618-1646) and -36.6 (nucleotides 1655-1682) kcal/mol) (16) were present downstream of the coding sequence. Of these structures, the preceding one 14 bp after the TGA stop codon was followed by a short T-rich sequence, which was thought to be probably active as the transcription termination signal.

Codon usage in the mdh gene is shown in Table II. The G+C content in the coding region of the mdh gene was found to be 68%, which is almost identical with the average G+C content of the total chromosomal DNA of T. flavus (68%) (1). Highly frequent use of G/C at the third letter of the codon was observed within the coding region.

Cloning of the Mutated mdh Gene and Identification of Its Mutation Point—A mutant strain, T. flavus F428, gave a 2 to 3 times higher enzyme activity in the crude extract. We cloned the mdh gene from the mutant strain by the colony hybridization method using the HindIII fragment which contained the whole native mdh gene as the probe. A recombinant plasmid pTM’1 carrying the mutant HindIII fragment on pBR322 was obtained, the construction of which was identical.
with that of pTA101. Crude extract of the E. coli 5131-5 transformant harboring pTM'1 showed a malate dehydrogenase activity about 3 times higher than that of E. coli 5131-5 (pTA101).

In order to identify the mutation point, hybrid plasmids between pTM'1 and pTA101 were constructed by using each of the unique BglII and SacI sites within the coding region of the mdh gene, and E. coli transformants harboring these hybrids were examined for their enzyme activity. As shown in Fig. 4, all the hybrid plasmids containing the BglII-SacI fragment of the mutated mdh gene conferred the elevated enzyme activity to the transformants. Sequencing of the BglII-SacI fragment revealed that a single nucleotide exchange of Thr-190 of the native enzyme to be replaced by isoleucine (Fig. 3).

Catalytic Properties of the Mutated Malate Dehydrogenase—

The mutated malate dehydrogenase was purified from E. coli 5131-5 (pTM'7) to give a single band upon SDS-polyacrylamide gel electrophoresis, and its enzymatic properties were examined. The native enzyme was also purified to homogeneity from E. coli 5131-5 (pTA101) and examined as control.

Double reciprocal plots of the oxalacetate reducing reaction catalyzed by the transformants are shown in Fig. 5. The inhibitory effect on the native enzyme by oxalacetate at concentrations higher than 50 μM was almost completely absent in the mutated enzyme. A marked increase in the Km value along with an increase in the Vmax value for oxalacetate were also observed with the mutated enzyme. The kinetic parameters of both the native and mutated enzymes for oxalacetate reduction and malate dehydrogenation reactions are summarized in Table III. A distinct shift toward a more acidic optimum pH for oxalacetate reduction and a slight decrease in the heat stability of the enzyme were also induced by the mutation (Fig. 6).

### DISCUSSION

When the amino acid sequence of Thermus malate dehydrogenase was compared with those of the porcine heart mitochondrial and cytoplasmic enzymes (17, 18) and the partial sequence of E. coli malate dehydrogenase (19), the significant homology (35%) was found with the porcine cytoplasmic enzyme (Fig. 7). Detailed structural analysis has revealed that a charge relay system between Asp-152 and His-180 in the porcine enzyme plays a central role in its catalytic function. It has been shown that 6 parallel β-sheets in the NH2-terminal region are contained in the nucleotide-binding domain and that several α-helices are involved in subunit interaction (17, 18). As shown in the alignment of amino acid sequences of both the enzymes, the NH2-terminal region and the sequence corresponding to the αC helix involved in subunit interaction (Glu-51–Leu-65) are well conserved in the Thermus enzyme. A homologous sequence was also found around the essential aspartic residue. His-187 of the Thermus enzyme may be assigned as the essential residue in view of its
to play some roles in interaction with substrates and cofactors as well as in the related dehydrogenases. Although the porcine malate dehydrogenase molecule has been assumed to be composed of a high G+C content, the coding region in the nucleotide sequence can be distinguished from the noncoding region by a high G+C content at the third position of the triplet codon (21). Applying this rule to the nucleotide sequence, the G+C contents at the third position of the mdh gene and the upstream unidentified reading frame are 96 and 98%, respectively. Although the E. coli mdh gene seems to be transcribed monocistronically (22), the mdh gene of Thermus may be composed of a polycistronic cluster and the promoter may be absent just upstream of the initiation codon of the mdh gene.

stream of the start codon of the mdh gene is present. In organisms like Streptomyces having DNA with a high G+C content, the coding region in the nucleotide sequence can be distinguished from the noncoding region by a high G+C content at the third position of the triplet codon (21).

Identical residues are enclosed by boxes.

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APPENDIX

FIG. 1. Restriction map and deletion derivatives of the cloned T. flavus DNA containing the mdh gene. The indicated regions of the DNA were inserted downstream of the anti-Tc' promoter of pBR322 or synthetic consensus promoter of pYEJ001, and the levels of malate dehydrogenase (MDH) activity directed by the plasmids were measured in the crude extracts. Solid areas indicate DNA from T. flavus AT-62 and hatched areas indicate DNA from a mutant F428. Clear areas indicate the extent of the deletions. Location of the identified mdh gene and direction of its amino acid sequence are indicated by the arrow. Cleavage sites for the restriction enzymes are designated as follows: H, HindIII; N, NcoI; X, XhoI; B, BamHI; Bg, BglII; Bs, BstEII; S, SacI; and Hf, Hinfl.

FIG. 2. Sequencing strategy for the cloned BamHI-XhoI fragment. All the regions indicated by unbroken arrows were sequenced by the method of Maxam and Gilbert (10). Those indicated by the dotted arrows were sequenced by both the Maxam and Gilbert method (10) and the M13 dideoxy method.
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FIG. 3. Nucleotide and deduced amino acid sequences of the mdh gene. The underlined amino acid sequences were determined by Edman degradation and sequencing. Two inverted repeat structures are indicated by facing arrows. The vertical arrow indicates the site of nucleotide exchange in the mutated mdh gene.