Most bacteria produce the dUMP precursor for thymine nucleotide biosynthesis using two enzymes: a dCTP deaminase catalyzes the formation of dUTP and a dUTP diphosphatase catalyzes pyrophosphate release. Although these two hydrolytic enzymes appear to catalyze very different reactions, they are encoded by homologous genes. The hyperthermophilic archaeon *Methanococcus jannaschii* has two members of this gene family. One gene, at locus Mj1102, encodes a dUTP diphosphatase, which can scavenge deoxyuridine nucleotides that inhibit archaeal DNA polymerases. The second gene, at locus Mj0430, encodes a novel dCTP deaminase that releases dUMP, ammonia, and pyrophosphate. Therefore this enzyme can singly catalyze both steps in dUMP biosynthesis, precluding the formation of free, mutagenic dUTP. Besides differing from the previously characterized *Salmonella typhimurium* dCTP deaminase in its reaction products, this archaeal enzyme has a higher affinity for dCTP and its steady-state turnover is faster than the bacterial enzyme. Kinetic studies suggest: 1) the archaeal enzyme specifically recognizes dCTP; 2) dCTP deamination and dUTP diphosphatase activities occur independently at the same active site, and 3) both activities depend on Mg²⁺. The bifunctional activity of this *M. jannaschii* enzyme illustrates the evolution of a superfamily of related enzymes that catalyze mechanistically distinct reactions.

Deoxyuridine nucleotides pose severe problems for cells. Although necessary precursors for thymine nucleotide biosynthesis, deoxyuridine nucleotides can interfere with DNA polymerase activity, either by inhibiting polymerases or by causing them to misincorporate 2'-deoxyuridine 5'-triphosphate (dUTP) in place of 2'-deoxycytidine 5'-triphosphate (dCTP) (1, 2). Ubiquitous DNA glycosylase enzymes remove uracil bases from DNA, which are otherwise formed by spontaneous cytosine deamination. The resulting apyrimidinic sites are cleaved by endonucleases, fragmenting cellular DNA (3).

At temperatures of 70–95 °C, rate constants for spontaneous cytosine deamination (in denatured DNA or cytidine nucleotides) are several orders of magnitude higher than those measured at more moderate temperatures (4). Therefore hyperthermophilic archaea that grow at such temperatures counter the mutagenic effects of cytosine deamination with a variety of thermostable uracil-DNA glycosylase enzymes (5). As a consequence of having efficient base excision repair mechanisms, these organisms must avoid misincorporating deoxyuridine nucleotides into DNA during replication by using highly discriminating DNA polymerases and by maintaining low dUTP levels in the cell (1, 6).

Eukaryotes, some bacteria and some archaea use zinc-dependent cytidine or deoxyctydine deaminase enzymes to produce uridine or deoxyuridine nucleotides. Yet many bacteria, including *Escherichia coli* and *Salmonella typhimurium*, produce most of their 2'-deoxyuridine 5'-monophosphate (dUMP) from 2'-deoxycytidine 5'-triphosphate (dCTP) using two different enzymes. These bacteria use dCTP deaminase (DCD, EC 3.5.4.13) to catalyze the nucleophilic substitution of a hydroxide ion for the 4-amino group of the cytosine base (7). A second enzyme, dUTP diphosphatase (DUT, EC 3.6.1.23), catalyzes the hydrolysis of the dUTP α-phosphorus anhydride bond to produce pyrophosphate (diphosphate) and dUMP, the obligatory precursor for thymidine nucleotides synthesis (8) (Fig. 1). Due to the toxicity of dUTP produced by DCD, the DUT enzyme is required to remove the accumulated product. Null mutations in the *E. coli* dut gene are lethal unless suppressed by null mutations in the *dcd* gene (9).

Despite catalyzing markedly different hydrolytic reactions, DUT and DCD proteins are encoded by homologous genes (9, 10). dUTP diphosphatase enzymes have been extensively studied, and four crystal structure models have been published for DUT proteins from *E. coli* (11), human (12), and two retroviruses (13, 14). However, only one DCD enzyme from *S. typhimurium* has been partially purified and characterized (15) and the *dcd* gene was subsequently identified in *E. coli* (9).

DUT proteins share five conserved signature motifs with the DCD proteins; four of these correspond to conserved uridine binding regions in DUT. These conserved domains have been used to identify dUTP diphosphatase homologs from diverse organisms (16) and to predict that two paralogous genes in the hyperthermophile archaeon *Methanococcus jannaschii* encode DCD and DUT proteins (10). The original annotation of the complete genome sequence from that organism described both gene products as dCTP deaminases because of their considerable sequence divergence from canonical DUT proteins (17).

In this work, we describe the characterization of two paralogous genes from *M. jannaschii* that are members of the *dut-dcd* family. Both genes were cloned in *E. coli*, and the heterologously expressed proteins were purified and characterized. The
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**Experimental Procedures**

**Materials**—All reagents were purchased from Sigma unless otherwise specified. Stock solutions of nucleotides were prepared in deionized water. Nucleotide concentrations were determined in sodium phosphate buffer (pH 7.2, 0.1 M NaCl) by UV spectroscopy using established molar extinction coefficients for cytidine nucleotides, uridine nucleotides, adenosine nucleotides, and guanosine nucleotides (18).

Cloning and Expression of the Mj0430 and Mj1102 Proteins in E. coli—The M. jannaschii genes at loci Mj0430 (Swiss-Prot accession number Q57872) and Mj1102 (Swiss-Prot accession number Q58520) were amplified by PCR from genomic DNA using oligonucleotide primers synthesized by Invitrogen. The Mj0430 gene was amplified using primers: Fwd, (5′-GGTGGTCATATGCTTCAATGGTAAACGAG-3′) and Rev, (5′-GATCCGAATTCCTTTTATGATTGTC-3′). The Mj1102 gene was amplified using primers: Fwd, (5′-GGTGGTCAATACTTCATGATTGATAAAG-3′) and Rev, (5′-GATCCGAATTCCTTTTATGATTGTC-3′). The forward primers introduced an NdeI restriction site at the 5′-end of the amplified DNA whereas reverse primers introduced a BamHI site at the 3′-end. PCR amplification was performed as described previously using a 50 °C annealing temperature for Mj0430 and a 55 °C annealing temperature for Mj1102 (19). PCR products were purified using a QIAquick spin column (Invitrogen) and then digested with NdeI and BamHI restriction enzymes (Invitrogen). Bacteriophage T4 DNA ligase (Invitrogen) was used to ligate the Mj0430 and Mj1102 genes into compatible sites in plasmids pET17b (Novagen) or pET7-7, respectively (20). DNA sequences were verified by dye-terminator sequencing at the University of Iowa DNA facility. The resulting recombinant plasmids were transformed into E. coli BL21-CodonPlus(DE3)-RIL (Stratagene) cells.

Transformed E. coli cells were grown in Luria-Bertani medium (200 ml) at 37 °C with shaking at 250 rpm and supplemented with 0.2% glucose and 100 µg/ml ampicillin. Cultures were harvested at 37 °C until they reached an absorbance at 600 nm of 0.8. Recombinant protein production was induced with 28 µl lactose. After an additional 2-h incubation with shaking at 37 °C, the cells were harvested by centrifugation (4000 × g, 5 min) and frozen at −20 °C. Induction of the desired proteins was confirmed by SDS-PAGE analysis of total cellular proteins.

Site-directed Mutagenesis—To test the possible function of conserved Asp135 and Glu145 residues of MjDCD-DUT, Mj0430 and Mj1102 genes were amplified by PCR from genomic DNA using oligonucleotide primers designed with instructions from the manufacturer's instructions with template plasmid pET17b. The primers were designed with instructions from the manufacturer's instructions with template plasmid pET17b. The primers were designed with instructions from the manufacturer's instructions with template plasmid pET17b. The primers were designed with instructions from the manufacturer's instructions with template plasmid pET17b. The primers were designed with instructions from the manufacturer's instructions with template plasmid pET17b.

**Measurement of dUTP Diphosphatase Activity—**dUTP diphosphatase activity was measured using a continuous spectrophotometric assay (15). In a thermostatted quartz cuvette, the following reaction mixture (1 ml) was equilibrated at 60 °C for 5 min: 50 µl TES/NaOH buffer (pH 7.5), 0.1 mM NaCl, 5 mM MgCl₂, 1 mM 2-mecaptoethanol (Fisher) and 50 mM dUTP. The reaction was initiated by addition of dCTP to a final concentration of 0.2 mM. The decrease in absorbance was as a result of deamination of cytosine compounds was measured at 284 nm with an UV-VIS recording spectrophotometer UV-160A (Shimadzu). The molar extinction coefficient difference between deoxyxycytidine and deoxyuridine compounds is 3.8 × 10⁻³ dm³ mol⁻¹ cm⁻¹ (15).

**Purification of MjDCD-DUT**—Heterologously expressed MjDCD-DUT protein was purified from soluble cell-free extract by heat treatment and chromatographic methods. E. coli cells (13 g, wet weight) were suspended in 25 ml of extraction buffer (20 mm Tris/HCl, 2 mM dl-dithiothreitol (DTT), pH 7.6) and sonicated in a 50-ml plastic tube using a Diastat (Bio-force Products, Westport, CT) sonicator driven by a WAVE Ultrasonic Processor (Sonics & Materials, Inc.) operating at 50% power output. Samples cooled by ice water were sonicated for 8 min with 3 s on/off intervals. Soluble cell-free extract was obtained after centrifugation at 20,000 × g for 15 min at 4 °C. The E. coli proteins were denatured by heating the soluble cell-free extract at 70 °C for 15 min, followed by centrifugation at 20,000 × g for 10 min at 4 °C to remove the insoluble material. Heat-stable cell extract (16 ml) was applied to a Mono Q HR anion-exchange column (1 × 8 cm; Amersham Biosciences) equilibrated with buffer A (20 mm Tris/HCl, pH 7.5). Pumps attached to the column were controlled by a Biologic HR workstation (Bio-Rad). Bound protein was eluted with a 45-ml linear gradient from 0 to 1 M NaCl in buffer A at a flow rate of 1.0 ml/min. Fractions containing dCTP deaminase activity were pooled and concentrated in a N₂-pressurized stirred cell with a YM10 ultrafiltration membrane (Millipore). Concentrated protein was applied to a Sephacryl S-200 HR size exclusion column (1.6 × 60 cm; Amersham Biosciences) equilibrated with buffer B (50 mM HEPES/NaOH, 0.15 M NaCl, 2 mM DTT, pH 7.2). MjDCD-DUT protein was eluted at a flow rate of 0.5 ml/min and was collected in 1-ml fractions. Fractions containing dCTP deaminase activity were pooled and concentrated in a N₂-pressurized stirred cell with a YM10 ultrafiltration membrane (Millipore) and then stored at −70 °C. Heterologously expressed Mj1102-encoded protein and the MjDCD-DUT D135N and MjDCD-DUT E145Q variants were purified by the same procedure.

Protein purity was evaluated by silver diamine staining of proteins separated by SDS-polyacrylamide gel electrophoresis (12% T, 4% C acrylamide) using a Tris/glycine buffer system. Sizes of the denatured proteins were determined by comparison to low molecular weight protein standards (Bio-Rad). Protein concentrations were measured using the Bradford total protein assay (Bio-Rad) with bovine serum albumin as a standard.

**Analytical Size Exclusion Chromatography—**Size exclusion chromatography was performed at room temperature on a Superose 12 HR column (1 × 30 cm; Amersham Biosciences). The column was equilibrated in buffer B and run with a flow rate of 0.4 ml/min. Protein standards used to calibrate the sizing column were horse spleen apoferritin (440,000), potato β-amylase (200,000), yeast alcohol dehydrogenase (150,000), bovine serum albumin (67,000), bovine erythrocyte carbonic anhydrase (29,000), and horse heart cytochrome c (12,400). Eluted protein was detected by their absorbance at 280 nm and/or dCTP deaminase activity.

**MALDI-TOF Mass Spectrometry of MjDCD-DUT Protein—**Purified MjDCD-DUT protein was analyzed by MALDI-TOF mass spectrometry. A saturated solution of 3-3-dimethoxy-4-hydroxycinnamic acid matrix was prepared in 50% (v/v) acetonitrile with 0.1% trifluoroacetic acid. Purified MjDCD-DUT (0.5 µl of a 2.2 mg/ml protein solution) was mixed with an equal volume of matrix solution on a stainless steel target (Kratos Analytical). Dried, crystalline samples were washed with 5 µl of 0.1% trifluoroacetic acid and dried under a stream of N₂ gas. Samples were analyzed in a Kratos KOMPACT SEQ MALDI-TOF instrument (Kratos Analytical) operated on the linear mode at 20 kV. For each sample, 50 spectra were collected by scanning across the sample. Average masses were identified using the combined sample set. Ions of sodium, matrix, bovine serum albumin, bovine pancreas insulin chain B (apoB), and horse heart cytochrome c were used for mass calibration.

**EPR Spectroscopy**—EPR spectroscopy was performed at 77 K in a nitrogen atmosphere using a Varian E-109 spectrometer. The EPR signal of the oxidized form of the enzyme was recorded using 20% 1,1-diphenyl-2-picrylhydrazyl (DPPH) as a reference. Protein concentrations were measured using a coupled enzyme assay. The coupling enzyme used was purified, heterologously expressed GTP cyclohydrolase from M. jannaschii, which has pyrophosphate phosphohydrolase activity, but has no effect on pyrimidine nucleotides (21). Inorganic phosphate was produced from pyrophosphate by including a thermostable coupling enzyme in the dUTP diphosphatase assay. The inorganic phosphate was measured from pyrophosphate released from dUTP was measured using an enzymatic assay in a pyrophosphate reagent kit (Sigma). Alternatively, inorganic phosphate was produced from pyrophosphate by including a thermostable coupling enzyme in the dUTP diphosphatase assay. The coupling enzyme used was purified, heterologously expressed GTP cyclohydrolase from M. jannaschii, which has pyrophosphate phosphohydrolase activity, but has no effect on pyrimidine nucleotides. Inorganic phosphate was then quantified by the malachite green dye-enhanced phosphomolybdate assay (21). Inorganic phosphate concentrations were calculated from a standard curve using 0–8 nM of KH₂PO₄ in 700 µl deionized H₂O. Absorances from reactions incu-
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**TABLE I**

| Stepa | Volume (ml) | Total protein (mg) | Total activity (μmol/min) | Specific activity (μmol/min/mg) | Yield (%) |
|-------|-------------|--------------------|---------------------------|-------------------------------|-----------|
| Crude extract | 28 | 810 | 2025 | 2.5 | 100 |
| Heat treatment | 16 | 104 | 634 | 6.1 | 31 |
| Mono Q | 11.4 | 54 | 443 | 8.1 | 22 |
| Sepharyl 200 | 8.9 | 33 | 347 | 10.5 | 17 |

* a MjDCD-DUT was purified from 13 g of *E. coli* cell paste.

Results

Identification, Expression, and Purification of MjDCD-DUT—dCTP deaminase/dUTP diphosphatase homologs have been identified in the genomes of several archaea (10). Two homologs, MJ0430 and MJ1102, were found in the complete genome sequences of *M. jannaschii* (17). However, it was not known whether one gene product functions as a dUTP diphosphatase and the other as a dCTP deaminase. We cloned both genes and expressed both proteins in *E. coli* for purification and characterization.

The recombinant MjDCD-DUT (encoded by MJ0430) was purified through heating, anion-exchange, and gel-filtration chromatography. Table I shows the purification of 33 mg of MjDCD-DUT from 13 g of *E. coli* cells. The specific activity of purified MjDCD-DUT was 10.5 μmol/min/mg. Enzyme specific activity increased 4.2-fold throughout the purification, which indicates the MjDCD-DUT constitutes 24% of the total *E. coli* soluble protein.

Analysis of the purified MjDCD-DUT by MALDI-TOF mass spectrometry showed a molecular mass of 23,619 ± 94 Da, which is close to its predicted 23,432 Da molecular mass from the genomic data. The denatured protein migrated on SDS-polyacrylamide gel an apparent molecular mass of 30,800 Da. From a Sepharose 12 HR size exclusion column, MjDCD-DUT eluted with an apparent molecular mass of 136,000 Da. This elution pattern suggests that native MjDCD-DUT protein may form a hexamer.

**MjDCD-DUT Catalytic Activities and Reaction Products**—When MjDCD-DUT was incubated with 0.2 mM dCTP at 60 °C, the enzyme not only catalyzed the deamination of dCTP, but also catalyzed the release of pyrophosphate to form dUMP. MjDCD-DUT can also catalyze the hydrolysis of dUTP to form dUMP and pyrophosphate. These results show that MjDCD-DUT is bifunctional: it catalyzes two consecutive reactions to form dUMP using dCTP as substrate. In contrast, *γ*-proteobacteria require separate dCTP deaminase and dUTP diphosphatase enzymes to catalyze the reactions (Fig. 1) (15).

Enzymes can catalyze pyrophosphate release by promoting the nucleophilic substitution by water at either the α- or β-phosphorus atoms of nucleotide triphosphates (24). To test the regiospecificity of MjDCD-DUT, the enzyme was incubated with dCTP in H₂¹⁸O, and the reaction products were converted to TMS derivatives. EI-MS analysis identified two compounds. The first corresponded to the (TMS)₄ derivative of pyrophosphate, M’⁺ = 466 m/z and M’⁻ = 451 m/z. Isotopic clusters of these ions had the same relative intensities observed for unlabeled pyrophosphate and showed no ¹⁸O incorporation. The second identified compound consisted of the (TMS)₂ derivative of dUMP M’⁻ = 509 m/z. The isotopic cluster of this ion showed the incorporation of two labeled oxygens each with 50% ¹⁸O enrichment. A fragment ion 299 m/z originating from the phosphate of the dUMP derivative contained only a single ¹⁸O confirming that one of the oxygens resided on the phosphate. The other oxygen was incorporated at the C-4 position of uracil during deamination. These data suggest that...
the phosphate anhydride cleavage proceeds by attack of activated water at the α-phosphate of 5'-nucleotide triphosphates.

**Characterization of MjDCD-DUT Activity**—The standard dCTP deaminase assay was used to characterize the purified MjDCD-DUT under various reaction conditions. When incubated with 0.1 M NaCl, the enzyme exhibited its maximum activity, compared with no NaCl (70%), 0.05 M (70%), 0.15 M (90%), or 0.2 M NaCl (90%) added. No change of enzyme activity was observed when MjDCD-DUT was assayed in 50 mM TES/NaOH (pH 7.5), 50 mM HEPES/NaOH (pH 7.5), or 50 mM MOPS/NaOH (pH 7.5) buffer. Nor did addition of 1 mM 2-mercaptoethanol and 1 mM DTT affect the activity, although 1 mM 2-mercaptoethanol was added in the standard assay.

Purified MjDCD-DUT is a thermostable enzyme. When heated at 90 °C for 10 min, MjDCD-DUT retained over 70% of its activity, assayed at 60 °C. Assays of MjDCD-DUT dCTP deaminase activity at various pH showed maximum activity at pH 7.5. As shown in Fig. 2, over the range from pH 6 to 9, the enzyme retains more than 50% of its maximum activity.

The substrate specificity of MjDCD-DUT was investigated using the standard deamination assay with 1 mM concentrations of other cytidine nucleotides. dCDP is a very poor substrate; MjDCD-DUT catalyzes its deamination and hydrolysis to form dUMP at only 4% of the rate of dCTP deamination. None of the following nucleosides or nucleotides was deaminated by MjDCD-DUT: dCMP, CTP, CDP, CMP, cytosine, or deoxycytidine. Neither ATP, dTTP, UTP, or GTP were substrates for MjDCD-DUT. Nor was pyrophosphate released in these assays. These results indicate that MjDCD-DUT is highly specific for dCTP as its substrate for deamination. The MjDCD-DUT activity requires metals. Divalent cation requirement was examined by assaying metal-free MjDCD-DUT. No activity was detected in reactions without added metals. Maximum dCTP deamination activity was observed with the addition of 2–5 mM MgCl₂ in the presence of 0.2 mM dCTP. No other divalent cations supported full activity at the concentration of 5 mM (Table II). Compared with its dCTP deaminase activity, dUTP diphosphatase activity of MjDCD-DUT showed a similar pattern of metal activation (Table II).

To test the inhibitory effects of other naturally occurring nucleotides on MjDCD-DUT dCTP deaminase activity, 1 mM concentrations of dCDP, dCMP, CTP, CDP, CMP, cytosine, or deoxycytidine, or any other cytidine nucleotide was added to the standard assay that included 0.2 mM dCTP. All of these nucleotides were inhibitors of the dCTP deamination activity of MjDCD-DUT. dTTP inhibited the activity by 80%.

**Table II**

| Added cation | Relative activity<sup>a</sup> | dCTP deamination | dUTP diphosphatase |
|--------------|-------------------------------|-------------------|-------------------|
| Mg<sup>2+</sup> | 100 | 100 |
| Mn<sup>2+</sup> | 80 | 62 |
| Ca<sup>2+</sup> | 75 | 68 |
| Ni<sup>2+</sup> | 65 | 100 |
| Ba<sup>2+</sup> | 15 | 21 |
| Zn<sup>2+</sup> | <3 | 5 |
| Cu<sup>2+</sup> | <3 | <2 |
| Ba<sup>2+</sup> | 0 | 11 |
| None | 0 | 0 |

<sup>a</sup> Relative activities are expressed as the percentage of activity compared with that obtained with 5 mM Mg<sup>2+</sup>. Assays of dCTP deamination and dUTP diphosphatase activities were performed as described under "Experimental Procedures."

To characterize the kinetic properties of MjDCD-DUT, initial rates for both dCTP deaminase and dUTP diphosphatase activities were measured at various substrate concentrations.
Figure 3 shows the amino acid sequences of MjDUT-DCD homologs on a black and white background. The relevant substrate for both reactions is likely a Mg\(^{2+}\)-substrate complex. Therefore, in these experiments, the Mg\(^{2+}\) concentration was fixed at 5 mM, more than a 5-fold excess over the substrate concentrations. Both sets of activity data were fit to the Michaelis-Menten first-order rate equation. At 60 °C, apparent kinetic parameters of MjDCD-DUT were determined, and $K_m = 263 \pm 64 \mu M$, $V_{max} = 23.9 \pm 2.6 \mu mol/min/mg$ for dUTPase activity.

Because both dCTP and dUTP are substrates for MjDCD-DUT, concentrations were chosen to give similar activities with 100% DUT diphosphatase activity, a competition assay was used to determine the substrate specificity. The combined activity for the two reactions was consistent with a general model for reactions catalyzed by DUTP diphosphatases (16). Crystal structure models of DUT (data not shown) (25).

The altered protein had an apparent molecular mass of 57,900 Da, measured by analytical gel-filtration chromatography, compared with a mass of 138,000 Da for the wild-type MjDCD-DUT. Thus the replacement of Asp\(^{135}\) may destabilize the protein’s quaternary structure.

In the analogous bacterial cytidine deaminase enzymes, the side chain of a conserved glutamate residue is proposed to act as a general acid/base catalyst, facilitating release of the leaving -NH\(_2\) group after a nucleophilic hydroxide ion attacks the substrate. Therefore the MJ1102 gene encodes a DUT-DCD homolog that lacks dCTP deaminase activity. Therefore the MJ1102 gene encodes a DUT-DCD homolog, but in few DUT homologs, we used site-directed mutagenesis to replace Glu\(^{145}\) with Gln (MjDCD-DUT E145Q). Purified MjDCD-DUT E145Q protein had no dCTP deaminase activity. Therefore the MJ1102 gene encodes a DUT-DCD homolog.

Characterization of MJ1102-encoded Protein—Purified MJ1102-encoded protein showed a single band on a SDS-polyacrylamide gel with an apparent molecular mass of 18,000 Da, compared with its predicted molecular mass of 18,640 Da. From the Sepharose 12 HR size exclusion column, the MJ1102-encoded protein showed a single band on a SDS-polyacrylamide gel with an apparent molecular mass of 18,640 Da, which suggests that the native protein may form a dimer. When incubated at 60 °C, it catalyzed hydrolysis of dUTP to dUMP and pyrophosphate. It had no detectable dCTP deaminase activity. Therefore the MJ1102 gene encodes a DUT diphosphatase.
homotrimeric DUT proteins show each active site is comprised of residues from motifs 2, 3, 4, or 5 contributed by all three subunits. We predict that the hexameric MjDCD-DUT protein of residues from motifs 2, 3, 4, or 5 contributed by all three homotrimeric DUT proteins show each active site is comprised of residues from motifs 2, 3, 4, or 5 contributed by all three subunits.

### Table III

| Source                          | $K_m$ (μM) | $k_{cat}$ (s$^{-1}$) | $K_m$ (μM) | $k_{cat}$ (s$^{-1}$) |
|---------------------------------|------------|----------------------|------------|----------------------|
| S. typhimurium (DCD)            | 300        | 0.35                 | ND         | ND                   |
| E. coli (DUT)                   | ND         | ND                   | ND         | ND                   |
| M. jannaschii (MJ1102)          | ND         | ND                   | ND         | ND                   |
| M. jannaschii (MJ1102)          | ND         | ND                   | ND         | ND                   |

*Enzymes and sources of their kinetic parameters were S. typhimurium dCTP deaminase (15), E. coli DUT (36), MjDCD-DUT, MjDCD-DUT E145Q, and MjDCD-DUT E145Q (this work). Turnover numbers are calculated by assuming one active site per protein subunit. The dependence of the rate of reaction on dCTP concentration was sigmoidal. The rate of the reaction was half saturated at about 300 μM dCTP concentration.

**DISCUSSION**

Previously, two pathways were known to convert deoxycytidine nucleotides to dUMP for thymidine biosynthesis. Eukaryotes and some microorganisms dephosphorylate deoxycytidine nucleotides to form dCMP and then deaminate the nucleotide monophosphate using a zinc-dependent deaminase (15, 29). This pathway does not produce dUTP, so the cells need only sequester dCMP and dUMP from pyrimidine nucleotide monophosphate kinases to avoid futile cycles. On the other hand, many bacteria use the DCD protein to deaminate dCTP and the DUT protein to rapidly hydrolyze dUTP to form dUMP. These organisms require DUT proteins with high affinity for dUTP to avoid accumulating that toxic intermediate (Table III). *M. jannaschii* has circumvented the problem of dUTP accumulation by directly coupling dCTP deamination to the release of pyrophosphate using the MjDCD-DUT enzyme described here. This strategy of sequestering dUTP is similar to the use of multifunctional, substrate channeling enzyme complexes by eukaryotes to catalyze the initial steps in pyrimidine biosynthesis (30, 31).

Like the *S. typhimurium* DCD protein, MjDCD-DUT activity is inhibited by dTTP (15). Although the mechanism of this inhibition is not yet clear, dTTP concentrations may regulate the pathway by feedback inhibition. While some DUT proteins are inhibited by dTTP, they hydrolyze dTTP and dCTP with much lower efficiency than dUTP (12). Therefore members of the DCD/DUT family effectively discriminate among pyrimidine nucleotides.

Having a bifunctional enzyme that sequesters the dUTP intermediate does not relieve *M. jannaschii* of the need to hydrolyze free dUTP produced by spontaneous deamination and pyrimidine nucleotide kinase activity. Therefore that organism also has a dedicated dUTP diphosphatase. Although *M. jannaschii* DUT has a higher affinity for dUTP than does MjDCD-DUT, its affinity is still much lower than that of the *E. coli* or human DUT enzymes (Table III). If *M. jannaschii* DUT has similar kinetics in vivo, then the cells may require relatively high concentrations of DUT to maintain low dUTP levels during DNA replication.

Although the order of the hydrolytic reactions has yet to be determined, MjDCD-DUT’s ability to hydrolyze dUTP to dUMP and pyrophosphate, but not to deaminate dCMP to dUMP, indicates that the reaction likely proceeds in the order of dCTP → dUTP → dUMP. Similar metal activation profiles for dCTP deaminase and dUTP diphosphatase activities, along with the results of a competition assay, suggest that deamination and pyrophosphate release occur at the same binding site. The fact that MjDCD-DUT E145Q protein has no deaminase activity but can still catalyze the release of pyrophosphate supports a model of both enzymatic reactions occurring at the same substrate binding site, but independently.

Both the affinity for dCTP and the turnover of MjDCD-DUT are much higher than those of the homologous *S. typhimurium* dCTP deaminase (Table III). In contrast to the cooperative kinetics observed for the *S. typhimurium* DCD enzyme, MjDCD-DUT kinetics show no sigmoidal relationship between dCTP concentration and the rate of deamination. On the other hand, both enzymes require divalent cation metals and the effective substrate for the enzymes is likely a Mg$^{2+}$-dCTP complex. Unlike the cytidine deaminase (26) or adenosine deaminase (32, 33), MjDCD-DUT does not use a zinc-activated hydroxide to attack C-4 of the cytosine ring. The analogous mechanism of hydroxide activation by MjDCD-DUT is not yet known; however, the enzyme may use either an activated Mg$^{2+}$-H$_2$O nucleophilic complex or the Glu$^{145}$ side chain carboxylate could serve as a general base, abstracting a proton from water. The loss of deamination activity in the MjDCD-DUT E145Q variant protein indicates that this residue is essential for deaminase activity. This glutamate residue is conserved in all DCD homologs, but not in most DUT homologs, except for crenarchaeal DUT proteins (Fig. 3). Therefore Glu$^{145}$ is necessary but may not be sufficient for deaminase activity. In addition, we found that residues in motif 5 of DCD proteins differ from those in DUT homologs. In DUT crystal structure models, positions of this glycine-rich carboxy-terminal domain vary. Only in substrate-bound human and viral DUT structures did this domain become ordered (in a closed enzyme conformation), positioning the residues near the active site (12, 34). Additional studies will be needed to determine the contribution of this domain to the MjDCD-DUT enzyme’s activities.

Unlike the bacterial dCTP deaminase, the MjDCD-DUT enzyme also has dUTP diphosphatase activity. Crystal structure models of DUT show that each of three active sites in the homotrimer is comprised of residues at the interface of all three subunits. One conserved acidic residue, equivalent to Asp$^{135}$ of
MjDCD-DUT, forms hydrogen bonds to tightly bound water molecules in the active site. It has been suggested that this residue is also involved in catalysis, activating water by proton abstraction (35, 36). A structural model of equine infectious anemia virus DUT shows that the analogous aspartate side chain forms a hydrogen bond with a main chain amide group from an adjacent subunit, stabilizing the protein’s oligomeric structure (14). The variant protein MjDCD-DUT D135N is unable to catalyze either the dCTP deaminase or the dUTP diphosphatase reaction. Because results from gel-filtration chromatography suggest that the altered protein does not adopt the same quaternary structure as the wild-type enzyme, the specific role of Asp \(^{135}\) remains to be determined.

A molecular phylogeny of the DUT and DCD homologs implies that dCTP deaminase activity evolved from a dUTP diphosphatase after a single gene duplication event (10). Although both dut and dcd genes have been vertically inherited in many organismal lineages, phylogenies indicate numerous instances of horizontal gene transfer, which may have been enabled by viral vectors (37). Yet these phylogenies did not explain the evolution of deaminase activity in DCD, presumably from an ancestral gene encoding a dUTP diphosphatase. The bifunctional activity of the MjDCD-DUT protein described here illustrates how a superfamily of enzymes sharing a common partial reaction has evolved into a suprafamily of enzymes that catalyze mechanistically distinct reactions (38).

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