

A Highly Active Protein Repair Enzyme from an Extreme Thermophile: The L-Isoaspartyl Methyltransferase from *Thermotoga maritima*¹

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We show that the open reading frame in the *Thermotoga maritima* genome tentatively identified as the *pcm* gene (R. V. Swanson *et al.*, *J. Bacteriol.* 178, 484–489, 1996) does indeed encode a protein L-isoaspartate (D-aspartate) O-methyltransferase (EC 2.1.1.77) and that this protein repair enzyme displays several novel features. We expressed the 317 amino acid *pcm* gene product of this thermophilic bacterium in *Escherichia coli* as a fusion protein with an N-terminal 20 residue hexa-histidine-containing sequence. This protein contains a C-terminal domain of approximately 100 residues not previously seen in this enzyme from various prokaryotic or eukaryotic species and which does not have sequence similarity to any other entry in the GenBank databases. The C-terminal region appears to be required for enzymatic function as no activity is detected in two recombinant constructs lacking this domain. Sedimentation equilibrium analysis indicated that the enzyme is monomeric in solution. The K_m values for measured for peptide and protein substrates were found to be intermediate between those of the high-affinity human enzyme and those of the lower-affinity wheat, nematode, and *E. coli* enzymes. The enzyme was extremely heat stable, with no loss of activity after 60 min at 100°C. Enzyme activity was observed at temperatures as high as 93°C with an optimal activity of 164 nmol/min/mg protein at 85°C. This activity is approximately 18-fold higher than the maximal activities of mesophilic homologs at 37°C. These

data suggest that the *Thermotoga* enzyme has unique features for initiating repair in damaged proteins containing L-isoaspartyl residues at elevated temperatures. © 1998 Academic Press

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Particular enzymes that have been conserved throughout evolution have been referred to as “first edition” proteins (1). These enzymes are thought to catalyze the essential pathways that have been conserved over the billions of years of evolutionary history that separate bacteria and humans. As new gene and genome sequences are reported for organisms representative of the deepest roots of the evolutionary tree, we begin to see what the “first printing” of these enzymes might have been like (2, 3).

One such enzyme, the protein L-isoaspartyl (D-aspartyl) O-methyltransferase (EC 2.1.1.77), has been identified in a wide variety of organisms, including mammals, nematodes, insects, plants, and bacteria (4–10) and catalyzes the transfer of a methyl group from S-adenosylmethionine (AdoMet) to the α -carboxyl group of L-isoaspartyl residues (11). These residues result from the spontaneous deamidation of asparaginyl residues and isomerization of aspartyl residues (12) and can compromise the structure and function of proteins due to the addition of a methylene group into the polypeptide backbone. The detrimental effects of isoaspartyl residues on enzymatic activity have been demonstrated in several proteins, including the bacterial phosphocarrier protein, HPr (13), calmodulin (14), and others (15–18). The methylation of these abnormal residues by the L-isoaspartyl methyltransferase has been

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shown, *in vitro*, to initiate the repair of these residues to aspartyl residues (13, 14, 19, 20). The best *in vivo* evidence for the beneficial effects of repairing isoaspartyl damage has come from mouse studies, where animals lacking this enzyme accumulate isoaspartyl damage and die of seizures at an early age (21). Furthermore, disruption of the *pcm* gene in the nematode *Caenorhabditis elegans* has also shown to have detrimental effects on dauer-phase survival and reproductive fitness (22). Finally, studies in *Escherichia coli* have shown that stationary-phase cells lacking the L-isoaspartyl methyltransferase have a greater susceptibility to oxidative and other stresses (23).

Swanson *et al.* identified an open reading frame in the thermophilic bacterium *Thermotoga maritima* that encodes a potential protein with amino acid sequence similarity to the protein L-isoaspartyl methyltransferase from *E. coli* (*pcm* gene product, 31.2% identity) and the human enzyme (25.5% identity) (24). These similarities are in regions common to all protein L-isoaspartyl methyltransferases (10, 25). To characterize this potentially novel type of L-isoaspartyl methyltransferase, we decided to overexpress this enzyme, purify it to homogeneity, and characterize its enzymatic activity. This protein methyltransferase is of interest because *T. maritima* thrives in a high temperature environment, growing optimally at 80°C and able to grow at temperatures as high as 90°C (26, 27), where the spontaneous rate of protein isomerization would be expected to be very high. Based on the energy of activation measured in model peptides, an increase in temperature from 23 to 90°C would increase the rate of isoaspartyl residue formation 910-fold (28). Thus, one of the challenges for *T. maritima* and other thermophiles is to avoid the accumulation of proteins with altered aspartyl and asparaginyl residues, and this methyltransferase may play an important role in this process.

MATERIALS AND METHODS

Construction of *Thermotoga maritima* L-isoaspartyl methyltransferase overexpression plasmids. Two oligonucleotide primers were initially designed based on the putative *pcm* gene sequence of Swanson *et al.* (24) to allow PCR amplification of the full-length gene from *T. maritima* genomic DNA. The 5' primer (TM1, 5'-AGGGGGATCA-TATGAGAGAAAAGCT-3') overlaps the ATG initiation codon (underlined) of the *pcm* gene and was designed to include an *Nde*I restriction site by changing two bases (shown in boldface type). The 3' primer (TM2, 5'-AATTCTAGACTGGTGGTCTGACTC-3') is complementary to nucleotides 52–77 past the stop codon of the putative *pcm* gene and also created an *Xba*I site by two nucleotide changes (boldface). PCR amplification was achieved using *T. maritima* genomic DNA, kindly provided by Dr. Ronald V. Swanson (Diversa Corp.) as a template in a total of 25 cycles. A single product was detected from this reaction on agarose gel electrophoresis with the expected size of approximately 1.0 kbp.

The 1.0-kbp PCR product was first purified by agarose gel purification using the GeneClean III kit isolation method (Bio101, Inc.).

The purified DNA fragment was first treated with the Klenow fragment of *E. coli* DNA polymerase I. After heat denaturation of the Klenow fragment, the 5'-end of the *pcm* gene was digested with *Nde*I. This fragment was purified away from restriction enzymes and small DNA fragments using GeneClean III kit and was ligated in between the *Nde*I and *Sma*I sites of pT7-7 using T4 DNA ligase (Gibco BRL) in a 10- μ l reaction for 12 h at 4°C. The products of the ligation reactions were transformed into *E. coli* strain DH5 α (Gibco BRL). Insert-containing plasmid clones were initially identified by detection of a single 3.5-kbp *Cl*aI-digested fragment on agarose gel electrophoresis. The plasmids (designated pTmPCM1) containing the full-length *pcm* gene were confirmed by further restriction digestions. Additionally, complete nucleotide sequencing of the *pcm* gene and 50 bp flanking either end of it in this plasmid showed that it was inserted correctly and had the expected sequence (24).

The *T. maritima pcm* gene from pTmPCM1 was then subcloned into a plasmid that contains the *E. coli pcm* gene fused to an additional sequence at the N-terminus encoding a hexa-histidine sequence and a thrombin cleavage site that facilitated purification. We chose to move the *T. maritima pcm* gene into a new plasmid rather than modifying pTmPCM1 because of an additional *Xba*I site in the *pcm* coding sequence. The parent plasmid, pJI19H (Fig. 1), was constructed by first replacing the *Xba*I to *Nde*I region in the vector portion of pJI19 with a synthetic 103-bp DNA fragment designed to introduce a ribosomal binding site sequence along with an initial 20 amino acid open reading frame that would be fused to any gene properly ligated into the *Nde*I site. This sequence is identical to the *Xba*I to *Nde*I region upstream of the multiple cloning site of the expression vector pET-15b (Novagen, Inc.). The plasmid pJI19 was digested with *Xba*I and *Nde*I and purified by agarose gel electrophoresis using the GeneClean III kit as described above. Finally, the oligonucleotide fragment containing the hexa-histidine leader coding sequence was ligated into pJI19 and transformed into DH5 α as described above. Plasmids bearing an insert were identified by restriction digestion and the resulting plasmid was designated pJI19H (Fig. 1).

The *E. coli pcm* gene from the pJI19H plasmid was removed by digestion with *Nde*I and *Cl*aI and purified by agarose gel purification as described above. A fragment containing the *T. maritima pcm* gene was isolated by digesting pTmPCM1 with *Nde*I and *Cl*aI followed by gel purification. The ligation of the *T. maritima pcm* gene into the modified pT7-7 vector resulted in plasmid pTmPCM2 (Fig. 1).

Two C-terminal deletion mutants of *T. maritima* methyltransferase were constructed to examine the role of the unique C-terminal domain. One mutant, lacking the 411 base pairs that code for the C-terminal domain, was constructed by digesting pTmPCM2 with *Xho*I and *Hind*III and religating to generate pTmPCM3 (Fig. 1). A second mutant was also constructed by the removal of a 461 nucleotide *Bam*HI to *Bam*HI portion of pTmPCM2 to generate pTmPCM4 (Fig. 1). After digestion with the appropriate enzymes, the DNA was treated with Klenow, and the DNA fragments were self-ligated to closed, circular plasmids and transformed into DH5 α . The modified methyltransferase amino-acid sequence encoded by the pTmPCM3 plasmid contains 241 amino acids, including the N-terminal poly-histidine 20-amino-acid sequence, and a C-terminal tetrapeptide sequence not found on the wild-type enzyme. The *pcm* gene sequence from the pTmPCM4 plasmid encodes a 237 amino-acid protein, including the poly-histidine sequence and a 25-residue C-terminal region not found on the wild-type enzyme (Fig. 1).

Bacterial growth. *E. coli* strains were grown in 2-liter flasks containing 1 liter of Luria-Bertani (LB) broth at 37°C with shaking at 250 rpm to provide aeration in a New Brunswick Innova 4300 incubator shaker. Methyltransferase overexpression was achieved by inoculating LB medium containing 50 μ g ampicillin and 20 μ g chloramphenicol per milliliter with an overnight culture of *E. coli* strain BL21 (DE3) (Novagen, Inc.) harboring the pLysS (Novagen, Inc.) plasmid (to reduce background expression) and one of the over-

TABLE I
Purification Summary

	Activity ^a (nmol/min/ml)	Total volume (ml)	Total activity (nmol/min)	Protein concentration ^b (mg/ml)	Specific activity (nmol/min/mg)	Yield (%)	Fold purification
Crude	4.2	116	489	30	.14	100	1.0
Heated supt	4.1	80	330	4.7	.87	67	6.2
100K supt	4.1	80	331	5.4	.76	67	5.4
DE52 pool	2.0	113	223	1.3	1.5	46	10.6
Ni-NTA pool	5.0	68	340	0.03	156	70	1110

^a A 10- μ l aliquot sample from each step in the purification was assayed in triplicate at 85°C for L-isoaspartyl methyltransferase activity as described under Materials and Methods.

^b Protein concentration was determined by the Lowry assay (30), which was preceded by TCA precipitation to remove any compounds which would interfere with the assay.

expression plasmids shown in Fig. 1. Strain BL21 (DE3)/pLysS was grown in LB medium containing 20 μ g chloramphenicol per milliliter as a control. Cell growth was monitored by the O.D._{600nm} of the culture. At an O.D._{600nm} value of 0.4, isopropyl thio- β -D-galactoside (IPTG, biotech grade, Fisher Biotech) was added to the culture to a final concentration of 1 mM to induce expression of the *T. maritima pcm* gene product. The cells were allowed to grow for another 2 h to achieve maximal expression, then the cells were harvested by centrifugation at 5000g for 20 min. The cells were washed twice in Buffer A (50 mM Tris-HCl, 5 mM disodium EDTA, 25 μ M PMSF, 15 mM β -mercaptoethanol, 10% glycerol, pH 7.0).

Preparation of cytosolic extract. All steps were performed at 4°C unless indicated otherwise. The cell pellet was resuspended in Buffer A at a ratio of 2 ml Buffer A per gram wet weight of cells. The cells were lysed by two passages through a French Press cell (American Instrument Co. Inc.) at 15,000 p.s.i. or sonicated for a total of 1 min at 15-s intervals with incubation on ice for 1 min between cycles. Unbroken cells and debris were sedimented at 27,000g for 30 min. The supernatant fraction, designated crude extract, was heated in a 500-ml glass beaker with a stir bar set to a low speed placed in an 80°C water bath with a thermometer to monitor the temperature. The extract was heated to 80°C over 4–5 min and then maintained at 80°C for 15 min with constant stirring and then placed in an ice-water bath until cooled to <10°C. The resulting slurry was transferred to centrifuge tubes and the beaker was washed with a small volume (1–2 ml) of Buffer A. The precipitate was removed by centrifugation at 27,000g for 30 min, and the supernatant was designated heated extract. The cytosolic extract obtained after further centrifugation at 100,000g for 1 h.

DEAE-cellulose fractionation of cytosolic extract. A DEAE-cellulose (DE-52, Whatman) column (112-ml bed volume) was equilibrated with Buffer A at a flow rate of 20 ml per hour. After the cytosolic extract (80 ml) was applied, the column was washed with 112 ml of Buffer A and then eluted with a gradient of 330 ml of Buffer A containing 0 to 500 mM NaCl. The column was equilibrated and eluted at room temperature and fractions (about 3 ml) were collected. The activity peak of fractions 63 to 100 (corresponding to about 50 to 200 mM NaCl) was pooled.

Nickel-nitrilotriacetic acid column fractionation. The activity pooled from the DE-52 column was extensively dialyzed to remove any trace amounts of EDTA. The nickel-nitrilotriacetic acid affinity

column (50-ml bed volume; Ni-NTA, Qiagen, Inc.) was equilibrated in Buffer B (50 mM sodium phosphate, 300 mM NaCl, 10 mM imidazole-HCl, pH 8.0) at a flow rate of 20 ml per hour. After loading the sample, the column was washed with 100 ml of Buffer C (50 mM sodium phosphate, 300 mM NaCl, 20 mM imidazole, pH 8.0). The proteins were eluted from the column with a linear gradient of 250 ml of 0–0.5 M imidazole-HCl, pH 8.0, in Buffer C. The column was equilibrated and run at room temperature and 3-ml fractions were collected. The activity peak was pooled from fraction 85 to 100 and dialyzed into 50 mM sodium phosphate, pH 6.1.

Methyltransferase assay. Methyltransferase activity was quantitated essentially as described (8). A typical assay consisted of enzyme, 50 mM sodium citrate (pH 6.0), 50 μ M L-isoaspartyl-containing peptide substrate (KASA (isoD) LAKY), and 25 μ M S-adenosyl-L-[methyl-¹⁴C]methionine (AdoMet, Amersham Life Sciences) in a final volume of 40 μ l, unless otherwise noted. After incubation for the indicated time and desired temperature, the tube was cooled in an ice-water bath, spun briefly to collect any condensation, and frozen in dry-ice to quench the reaction. Immediately after thawing, 40 μ l of 0.2 M NaOH and 1% (w/v) SDS were added and mixed. A 60- μ l aliquot was then spotted onto a 1.9 \times 9.0 cm piece of thick filter paper (No. 1650962, Bio-Rad) prefolded into an accordian pleat and then inserted into the neck of a scintillation vial containing 5.0 ml of scintillation cocktail (Safety Solve, Research Products International Corp.). After 2 h at room temperature, during which time [¹⁴C]methanol produced from the hydrolysis of methyl esters diffuses into the fluor, the filter paper was removed and the vials were counted in an LS6500 Beckman Scintillation counter. Additional assays, where the peptide substrate was omitted, were also used as a control for background reactions and these values were subtracted from the reactions which included peptide. Peptide substrates were prepared by the UCLA Peptide Synthesis Facility; ovalbumin (Grade V, minimum 98% pure) was a Sigma Chemical Co. product.

Sedimentation velocity and equilibrium centrifugation. All runs were performed at 20°C on a Beckman Optima XL-A analytical ultracentrifuge using absorption optics at 280 nm and a double sector cell. The sample was in 300 mM NaCl, 50 mM sodium phosphate buffer, pH 7.4. Sedimentation equilibrium profiles were measured at 10,000 and 15,000 rpm and the data fitted with a nonlinear least-squares exponential fit for a single ideal species, with a baseline correction determined by overspeeding the rotor to deplete mac-

FIG. 1. Maps of plasmids used in this study. Plasmid constructions are described under Materials and Methods. The vector sequence of each plasmid is pT7-7 (29). Highlighted nucleotide and amino acid sequences are shown for relevant segments. The boxed amino acid sequences in pJI19H, pTmPCM2, pTmPCM3, and pTmPCM4 denote those sequences not found on the wild type L-isoaspartyl methyltransferase.

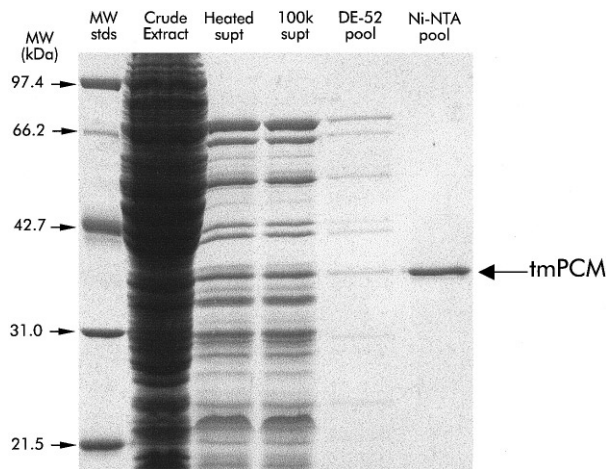


FIG. 2. SDS-PAGE analysis of protein L-isoaspartyl methyltransferase purification. A sample from each step in the purification was loaded into each lane of a 10% SDS-PAGE gel (31). Lane 1, molecular weight standards (1 μ g each, Bio-Rad, including phosphorylase b (97.4 kDa), bovine serum albumin (66.2 kDa), hen ovalbumin (42.7 kDa), bovine carbonic anhydrase (31.0 kDa), soybean trypsin inhibitor (21.5 kDa)); lane 2, 27,000g crude extract (300 μ g protein); lane 3, heat precipitated supernatant (20 μ g protein); lane 4, 100,000g supernatant (20 μ g protein); lane 5, pooled DE-52 activity peak (1.5 μ g protein); lane 6, pooled Ni-NTA activity peak (1 μ g protein).

romolecules from the meniscus, followed by reduction to 10,000 rpm and measurement of the baseline. A partial specific volume of 0.742 was calculated from the amino acid composition.

RESULTS AND DISCUSSION

Identification of L-isoaspartyl methyltransferase activity of the T. maritima pcm gene product expressed in E. coli. We hypothesized that the *T. maritima* methyltransferase should be active at a higher temperature than the endogenous *E. coli* enzyme. Therefore, we

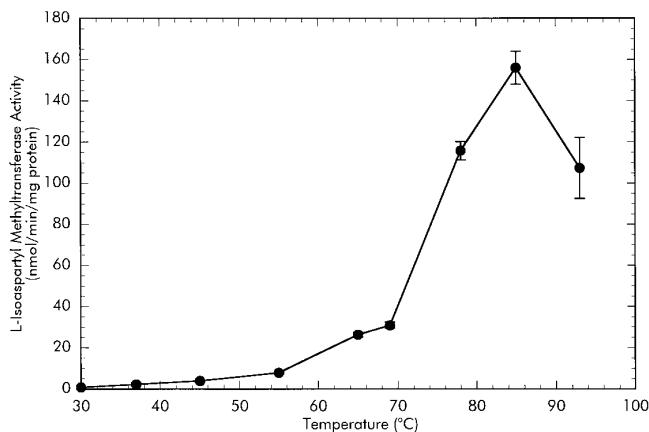


FIG. 3. Optimal temperature of *T. maritima* protein isoaspartyl methyltransferase. Identical assay mixtures were incubated at the indicated temperatures for 10 min. The procedure for the methyltransferase assay was as described under Materials and Methods.

assayed crude extracts of both the putative *T. maritima* L-isoaspartyl methyltransferase overexpressing strain (BL21 (DE3)/pLysS/pTmPCM1) and the parent *E. coli* strain (BL21 (DE3)/pLysS) for enzyme activity at 55°C. We found that while extracts of the control strain, containing only the *E. coli* methyltransferase, displayed little or no activity (0.3 ± 0.6 pmol/min/mg protein), extracts of the *T. maritima pcm* overexpressing strain gave an activity of 100 ± 26 pmol/min/mg protein, a value about 100 times that expected for an *E. coli* extract at 37°C (8). This result indicated that the putative *pcm* gene from *T. maritima* indeed encodes an L-isoaspartyl methyltransferase and we decided to purify this enzyme for further characterization.

Purification of the polyhistidine-tagged L-isoaspartyl protein methyltransferase from T. maritima. The *Thermotoga* methyltransferase was overexpressed in a total of 10 liters of *E. coli* cells harboring pTmPCM2. This plasmid encodes a fusion enzyme with an additional 20 amino acids at the N-terminus, including a hexa-histidine sequence as described under Materials and Methods (Fig. 1). We took advantage of the potential thermostability of the *T. maritima* L-isoaspartyl

TABLE II
Kinetic Constants of the *T. maritima* Protein L-Isoaspartyl O-Methyltransferase^a

Substrate	K_m (μ M)	V_{max} (nmol/min/mg)	k_{cat}/K_m ($mM^{-1} s^{-1}$)
AdoMet (85°C)	5.6 (± 1.1)	143 (± 7.3)	16.0
AdoMet (37°C)	1.9 (± 0.7)	1.7 (± 0.1)	0.6
KASA (isoD) LAKY (85°C)	25 (± 9.0)	164 (± 29.0)	4.0
KASA (isoD) LAKY (37°C)	2.8 (± 0.24)	2.3 (± 0.06)	0.5
VYP (isoD) HA (85°C)	33.6 (± 1.0)	184 (± 5.4)	3.3
VYP (isoD) HA (37°C)	4.1 (± 0.71)	1.8 (± 0.07)	0.3
Ovalbumin (37°C)	326 (± 56.0)	5.3 (± 0.4)	

^a Kinetic values of the *T. maritima* methyltransferase were measured as described under Materials and Methods. Each substrate was assayed at 12 to 14 different concentrations at 85 or 37°C for 10 min, except for ovalbumin, which was only measured at 37°C because the protein was prone to precipitation at 85°C. The ranges of substrate concentrations used for each of the methyl-accepting substrates were 0–10 mM KASA (isoD) LAKY peptide, 0–1.0 mM VYP (isoD) HA peptide, and 0–475 μ M ovalbumin. The K_m for the methyl donor AdoMet was determined using 50 μ M KASA (isoD) LAKY and AdoMet at concentrations between 2.5 and 120 μ M. Each point was also measured without peptide to determine background activity. Data plotting and curve fitting was accomplished by linear regression using the DeltaGraph 4.01 software (DeltaPoint, Inc.) on an Apple Power Macintosh computer. Mean values from 4 to 6 separate determinations are the reported V_{max} and K_m values and the standard error is in parentheses.

TABLE III

Comparisons of Michaelis–Menten Rate Constants for the Protein L-Isoaspartyl Methyltransferase from Various Organisms

Organism	Michaelis–Menten substrate constants (K_m)			
	KASA (isoD) LAKY (μM)	VYP (isoD) HA (μM)	AdoMet (μM)	ovalbumin (μM)
<i>T. maritima</i> ^a	2.8	4.1	1.9	326
<i>E. coli</i> ^b	50.6	11.8	N/A	727
Wheat germ ^c	12.7	21.7	N/A	>1500
<i>C. elegans</i> ^d	9.12	19.4	3.1	>3000
Human ^e	0.52	0.29	2.0	35

^a *T. maritima* methyltransferase values, which were taken from Table IV, are those measured at 37°C.

^b *E. coli* values were measured at 37°C using partially pure enzyme (8).

^c Wheat germ values were measured at 25°C using purified enzyme (7).

^d Values at 30°C using partially purified recombinant enzyme (9).

^e The values for the human methyltransferase purified from erythrocytes were measured at 37°C (35). N/A, data not available.

methyltransferase and achieved a sixfold purification by heat-precipitating the majority of *E. coli* proteins in the crude extract (Table I). The cytosolic extract was then fractionated on a DE-52 column. We found that a single peak of activity eluted at approximately 100 mM NaCl. The dialyzed pool of activity was then applied to a Ni-NTA affinity column to isolate the hexa-histidine tagged polypeptides. A peak of activity eluted from the column at an approximate imidazole concentration of 100 mM. SDS–PAGE analysis of the Ni-NTA pooled peak fractions revealed a single major polypeptide species with a molecular mass of approximately 35,000 Da, which correlates with the *T. maritima* methyltransferase calculated molecular mass from the translated amino acid sequence of 36,403 Da (Fig. 2).

Overall, we achieved an 1110-fold purification, with a recovery of 70% of the total enzyme activity (Table I). We estimate that the total amount of soluble enzyme present in the original culture was 0.3 mg of protein per liter of culture. Much of the expressed methyltransferase may be insoluble and present in inclusion bodies, since a large band of approximately the same molecular mass as the soluble methyltransferase was observed in the crude lysate pellet fraction by SDS–PAGE analysis (data not shown).

Characterization of the L-isoaspartyl methyltransferase from T. maritima. The optimal temperature for the *T. maritima* methyltransferase activity is 85°C (Fig. 3), which is in accord with the optimal growth temperature of 80°C for this organism (26). Significantly, this methyltransferase is only 1.5% as active at 37°C as it is at 85°C, with a specific activity of 2.3 nmol methyl groups transferred/min/mg protein. This activity is, however, only about fourfold lower than that of mesophilic methyltransferases assayed under similar conditions (7, 8, 11). A number of other thermophilic enzymes also are less active than their mesophilic

counterparts when assayed in the temperature range of the latter enzymes (32–34). Importantly, the specific activity of the *T. maritima* enzyme at 85°C (164 nmol/min/mg) is at least an order of magnitude larger than the specific activities of the mesophilic enzymes measured at 25 to 37°C (7, 8, 11).

We then measured the K_m values of the *T. maritima* methyltransferase for the methyl donor AdoMet and a variety of methyl-accepting substrates at 85°C. We found that the enzyme had a relatively high affinity for the methyl-accepting peptide substrates KASA (isoD) LAKY and VYP (isoD) HA with K_m values of 25 and 33.6 μM , respectively, as well as for AdoMet with a K_m value of 5.6 μM (Table II). When these values were measured at 37°C, the affinity for AdoMet was about 3-fold greater and the affinity for the methyl-accepting peptides were even 8- to 9-fold greater. Thus, the increased affinity for substrates at 37°C partially compensates for the lower V_{max} values at this temperature

TABLE IV
Thermal Stability of the *T. maritima* L-isoaspartyl Methyltransferase

Preincubation (°C)	Methyltransferase activity ^a (nmol/min/mg)
–80	49.8 (± 0.12)
4	38.4 (± 0.54)
37	54.0 (± 0.71)
85	53.0 (± 7.1)
100	56.0 (± 0.21)

^a Samples of purified L-isoaspartyl methyltransferase were preincubated at the temperature indicated for 1 h. In duplicate reactions, a 10- μl aliquot was added to a tube containing the premixed reaction components (with and without peptide) as described under Materials and Methods and incubated at 85°C for 20 min.

and the ratio k_{cat}/K_m differs by only 8- to 27-fold between the temperatures of 85 and 37°C for these substrates (Table II). We then compared the affinities of the *Thermotoga* enzyme for AdoMet and methyl-accepting substrates at 37°C with those of other protein L-isoaspartyl methyltransferases including enzymes from *E. coli*, wheat, nematodes, and humans (Table III). We found that the *T. maritima* methyltransferase displays the lowest K_m values (and thus highest affinities) for each of the methyl-accepting substrates examined at 37°C (including the protein ovalbumin) of all the nonmammalian methyltransferases (Table III). The kinetic parameters of the previously studied non-human enzymes have been shown to be fairly similar, while the human enzyme displays significantly higher affinities for peptide and protein substrates. The K_m value for the methyl donor AdoMet was similar in the *T. maritima*, human, and nematode enzymes. Since the efficiency of the repair reaction is directly related to both the activity of the methyltransferase and its affinity for its methyl-accepting substrates (11, 35, 36), these results suggest that the *Thermotoga* enzyme has been adapted for the higher expected load of protein damage that would occur at higher temperatures.

The human enzyme can also methylate D-aspartyl residues in proteins and peptides that arise from the racemization of the succinimide intermediate (11, 36). However, we were not able to detect any methylation of D-aspartyl-containing peptides by the *T. maritima* methyltransferase (data not shown).

The *T. maritima* methyltransferase was also found to be very resistant to thermal denaturation. The enzyme was able to withstand a 1-h preincubation at various temperatures, ranging from freezing in liquid nitrogen (−80°C) to heating in a boiling water bath (100°C), with no apparent loss of activity (Table IV). The pH activity profile of the *T. maritima* methyltransferase demonstrated a broad peak with a pH optimum of 5.6, with over 50% of maximal activity over the range of pH 5 to pH 8.5. The optimal pH value is very close to the pH optimum for the *E. coli* methyltransferase of pH 5.5 (37), but lower than that observed for the rat (pH 6–7) (38) or wheat methyltransferases (pH 7.5) (7). These results suggest that the enzyme may be very useful in biotechnological applications, including serving as an efficient probe of the accumulation of L-isoaspartyl residues in peptides and proteins under denaturing conditions.

A unique feature of the *T. maritima* methyltransferase is the C-terminal extension of approximately

100 amino acids. Whereas the largest previously characterized L-isoaspartyl methyltransferases is 230 residues, with the C-terminal residue no further than 12 residues from the position of the conserved leucine residue in post motif-III (10), the *T. maritima* enzyme is 317 residues with the C-terminal isoleucine residue spaced 109 residues past the conserved leucine residue (Fig. 4). This extended C-terminal sequence is not found in the putative L-isoaspartyl methyltransferase sequences from the thermophilic organisms *Methanococcus jannaschii* or *Archaeoglobus fulgidus* (see below). No other sequence containing this region was identified when the last 100 amino acids was queried against the GenBank databases. Two C-terminal deletion mutants of the methyltransferase was constructed in which the last 100 or 126 amino acids were removed (pTmPCM3 and pTmPCM4, respectively, Fig. 1). The mutant forms displayed no activity at either 80 or 37°C; thus, the C-terminal “domain” or portions of it appear to be required for either activity or stability. It is also possible that this domain is required for folding in the heterologous host.

We considered that a possible role for the extended C-terminal region of the protein would be to provide an oligomerization domain. Several studies have shown examples of enzymes where thermophilic versions operate as multimers and their mesophilic counterparts operate as monomers (40, 41). However, equilibrium centrifugation at 20°C revealed that the *T. maritima* methyltransferase protein is monomeric, with a calculated molecular mass of 34,000 Da, close to that expected for the polypeptide chain itself.

Comparison of genomic organization of the E. coli and T. maritima pcm genes. The *T. maritima pcm* gene, encoding the L-isoaspartyl methyltransferase, is located 700 nucleotides from the 5' end of the *cheA* gene and is transcribed in the opposite orientation (24). In *E. coli*, the *pcm* gene is located at 61 min on the chromosome and is cotranscribed with at least one other gene, *surE*, which is located directly upstream of *pcm*. We identified an open reading frame in *T. maritima* with 34.8% similarity to the *surE* gene of *E. coli*. This gene is not found in an operon with the *pcm* gene as it is in *E. coli* but, in fact, is found in an operon with the *def* gene, encoding a polypeptide deformylase. Although the function of SurE is unknown, initial evidence suggests that it may play some role in preventing the accumulation of isoaspartyl residues or other damage in the cytosolic proteins of *E. coli* (42). We

have also identified a sequence in *T. maritima* downstream of the *def* gene with significant similarity to the *nlpD* lipoprotein gene, which is located between *pcm* and *rpoS* at 61 min on the *E. coli* chromosome (43). This conserved organization of the *surE* and *nlpD* genes has been observed in many Bacterial species (data not shown; cf. Ref. 44).

Sequence analysis and phylogenetic comparisons. The *T. maritima* methyltransferase displays 31.2% amino acid sequence identity to the *E. coli* methyltransferase (Fig. 4) and 25.5% amino acid identity to the human methyltransferase (10). Database searches of both completed and partially completed genome sequences from prokaryotic sources have identified open reading frames that are also likely to code for L-isoaspartyl methyltransferases. These putative methyltransferases are found in species from both the Bacteria and Archaea. With the exception of *Haemophilus influenzae*, orthologs of the L-isoaspartyl methyltransferase have been found in all of the Gram-negative bacteria in the gamma subdivision. No sequences with identities to this methyltransferase have been found in the Gram-positive bacteria *Mycoplasma genitalium*, *Mycoplasma pneumoniae*, or *Bacillus subtilis*. These data correlate well with a previous study that assayed for methyltransferase activity and probed genomic DNA by hybridization for the presence of the *pcm* gene in several bacteria (45), except in the case of *Sinorhizobium meliloti*, where neither enzyme activity nor DNA hybridization was detected but a putative homologue was identified by sequence identity. No sequence with any similarity to the methyltransferase protein sequence was identified in the bacterium *Aquifex aeolicus* (Ron Swanson, personal communication) or in the spirochaetes *Borrelia burgdorferi* or *Treponema pallidum*. Putative L-isoaspartyl methyltransferases from 2 thermophilic species in the Archaeal domain, *Archaeoglobus fulgidus* (PcmA, Fig. 4) and *Methanobacterium thermoautotrophicum*, had the highest amino-acid identity to the *T. maritima* methyltransferase at 39.5 and 33.7%, respectively (Fig. 4). We also identified a second amino-acid sequence in *A. fulgidus* (PcmB, Fig. 4) that has 32.6% similarity to the *T. maritima* methyltransferase sequence.

The L-isoaspartyl methyltransferase is a good model enzyme to study phylogenetic relationships because the biochemistry of this widely distributed enzyme is well characterized. Phylogenetically, based on the L-isoaspartyl methyltransferase sequences from other prokaryotic species, we find that the *T. maritima* enzyme is the closest sequence to the divergence point between the Bacterial and Archaeal branches, which is in accord with similar analysis using 16S rRNA (3). Characterization of Pcm from Archaeal species will also give greater insight into the evolution of this pro-

tein repair enzyme. Comparative studies of the L-isoaspartyl methyltransferase may also yield greater insight into the properties of thermostable enzymes. The features that provide heat stability for the *T. maritima* methyltransferase may be distinct from those from other thermostable L-isoaspartyl methyltransferases since the C-terminal domain is not observed in the putative methyltransferases from thermophilic Archaea.

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