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Protein Repair Methyltransferase from the Hyperthermophilic Archaeon *Pyrococcus furiosus*

UNUSUAL METHYL-ACCEPTING AFFINITY FOR D-ASPARTYL AND *N*-SUCCINYL-CONTAINING PEPTIDES*

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Protein L-isoaspartate-(D-aspartate) *O*-methyltransferases (EC 2.1.1.77), present in a wide variety of prokaryotic and eukaryotic organisms, can initiate the conversion of abnormal L-isoaspartyl residues that arise spontaneously with age to normal L-aspartyl residues. In addition, the mammalian enzyme can recognize spontaneously racemized D-aspartyl residues for conversion to L-aspartyl residues, although no such activity has been seen to date for enzymes from lower animals or prokaryotes. In this work, we characterize the enzyme from the hyperthermophilic archaebacterium *Pyrococcus furiosus*. Remarkably, this methyltransferase catalyzes both L-isoaspartyl and D-aspartyl methylation reactions in synthetic peptides with affinities that can be significantly higher than those of the human enzyme, previously the most catalytically efficient species known. Analysis of the common features of L-isoaspartyl and D-aspartyl residues suggested that the basic substrate recognition element for this enzyme may be mimicked by an N-terminal succinyl peptide. We tested this hypothesis with a number of synthetic peptides using both the *P. furiosus* and the human enzyme. We found that peptides devoid of aspartyl residues but containing the *N*-succinyl group were in fact methyl esterified by both enzymes. The recent structure determined for the L-isoaspartyl methyltransferase from *P. furiosus* complexed with an L-isoaspartyl peptide supports this mode of methyl-acceptor recognition. The combination of the thermophilicity and the high affinity binding of methyl-accepting substrates makes the *P. furiosus* enzyme useful both as a reagent for detecting isomerized and racemized residues in damaged proteins and for possible human therapeutic use in repairing damaged proteins in extracellular environments where the cytosolic enzyme is not normally found.

Protein L-isoaspartyl-(D-aspartyl) *O*-methyltransferase (EC 2.1.1.77) is a repair enzyme that catalyzes the *S*-adenosylmethionine (AdoMet)-dependent[1] methyl esterification of the α-carboxyl group of L-isoaspartyl residues that originate from the spontaneous degradation of aspartic acid and asparagine residues in proteins (1–5). The enzyme-mediated methylation reaction is followed by nonenzymatic steps that result in the net conversion of L-isoaspartyl residues to L-aspartyl residues, representing a potentially important mechanism for avoiding the accumulation of damaged proteins as cells age (4–9). This methyltransferase is found in a wide array of organisms including eubacteria (10), plants (11, 12), nematodes (13), insects (14), and mammals (15). Its amino acid sequence is highly conserved (16). Its functional importance to the bacterium *Escherichia coli*, the nematode worm *Caenorhabditis elegans*, and mice has been assessed by analyzing the effect of knockout mutations of its structural genes. In *E. coli*, methyltransferase-deficient cells are more sensitive to stress in the stationary phase (17), whereas knockout worms show poorer survival in the dauer phase (18). Methyltransferase-deficient mice suffer fatal seizures at an early age (19–21). Interestingly, the effect of methyltransferase loss on the accumulation of damaged substrates in these knockout organisms is quite variable, from small effects seen in worms (22) and bacteria (23) to relatively large effects in mice (21).

Although all of the L-isoaspartyl methyltransferases characterized so far recognize L-isoaspartyl residues in synthetic peptide substrates, their relative affinity for these methyl-acceptors is also quite variable. For example, the human enzyme recognizes substrates with 40–1000 times the affinity of the *E. coli,* nematode, and plant enzymes (24). The functional significance of these differences is not clear, although mathematical simulations of the repair reaction clearly show that repair efficiency is directly related to the affinity of the enzyme for the methyl-acceptor (25). It is possible that the complexity of human protein interactions necessitates a more efficient repair to minimize the presence of proteins containing abnormal residues (21, 22).

In mammalian cells, this methyltransferase can also catalyze the AdoMet-dependent methylation of substrates containing D-aspartyl residues arising from spontaneous protein racemization reactions (15, 26, 27). The K_m for corresponding synthetic peptides containing D-aspartyl residues in place of L-isoaspartyl residues can be 700–10,000-fold higher, however. Additionally, no methylation of D-aspartyl peptides has been detected for the *E. coli* (28), worm (13), and higher plant (24) enzymes. In mammalian cells, it is possible that the methylation of D-aspartyl residues can lead to eventual L-aspartyl formation in a repair reaction similar to that observed for L-isoaspartyl residues, although considerable amounts of D-isoaspartyl residues would also be expected to accumulate (27).

We have been interested in comparing the properties of protein repair methyltransferases from a variety of organisms

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[1] The abbreviations used are: AdoMet, *S*-adenosyl-L-methionine; AdoHcy, *S*-adenosyl-L-homocysteine; [^{14}C]AdoMet, *S*-adenosyl-[*methyl*-^{14}C]-L-methionine; MTA, 5′-deoxy-5′-methylthioadenosine; HPLC, high pressure liquid chromatography.

that face distinct environmental challenges. We have been particularly interested in the enzymes that exist in thermophilic organisms where the rate of spontaneous isomerization and racemization would be expected to be greatly enhanced. Characterization of the enzyme from the eubacterium *Thermotoga maritima* demonstrated superior recognition of methyl-accepting substrates compared with the *E. coli*, plant, and worm enzymes, but the recognition is still 5–14-fold poorer than that of the human enzyme (10, 24). Additionally, the *T. maritima* enzyme has not been observed to methylate D-aspartyl-containing peptides recognized by the human enzyme (10). The three-dimensional structure of the *T. maritima* methyltransferase complexed with the product AdoHcy has been determined (29), but unfortunately does not shed light on the problem of the differential recognition of substrates by these enzymes.

We have recently been able to determine the structure of an L-isoaspartyl methyltransferase from a second thermophile, the hyperthermophilic archaebacterium *Pyrococcus furiosus* in complexes with a number of cofactors and an L-isoaspartyl peptide (30). *P. furiosus* was originally isolated from shallow geothermal marine sediments in Italy (31) and grows at temperatures ranging from 70 to 103 °C (32). In our biochemical characterization of the purified recombinant enzyme, we were surprised to find that the affinity of this enzyme for L-isoaspartyl-containing methyl-accepting substrates is as good or even better than the human enzyme, suggesting that it may have evolved for efficient repair under conditions where protein degradation may be exceptionally rapid. Significantly, we found that this enzyme is even better than the human enzyme in its ability to catalyze D-aspartyl methylation. By analyzing the common structural features of D- and L-isoaspartyl peptides, we propose that a minimal recognition element would be an *N*-succinyl peptide, and we now present data that this is indeed the case.

EXPERIMENTAL PROCEDURES

Recombinant Methyltransferases—*P. furiosus* recombinant L-isoaspartyl methyltransferase was prepared in *E. coli* cells as described (30). Using PCR primers based on the PF1922 gene sequence (complement of nucleotides 1,772,562–1,773,380, Utah Genome Center, *www.genome.utah.edu/Pfu102000.gb*), we amplified genomic DNA to produce a construct encoding an additional six histidine resides at the C terminus. This DNA was incorporated into the plasmid pTrcHis2-TOPO and used to transform *E. coli* TOP10 cells. Protein expression was induced with isopropyl thio-β-D-galactoside treatment, and the enzyme was purified by chromatography on a nickel-containing HiTrap column of a heat-treated soluble cellular extract as described (30). The purified protein consists of a single polypeptide of 24 kDa on SDS gels. From densitometric analyses of overloaded samples on these gels, we estimate the polypeptide purity to be at least 98%. Automated Edman analysis gave a single N-terminal sequence of MHLYS (30). The enzyme has a specific activity at pH 7.5 of about 12 nmol methyl groups transferred/min/mg protein at 68 °C and 27 nmol/min/mg protein at 85 °C using VYP-L-*isoAsp*-HA as methyl-acceptor as assayed below. The human recombinant type II L-isoaspartyl methyltransferase was prepared according to MacLaren and Clarke (33).

Methyl-accepting Substrates—The peptides VYP-L-*isoAsp*-HA and KASA-L-*isoAsp*-LAKY were synthesized and HPLC-purified by California Peptide Research Inc. (Napa, CA). KASA-D-*Asp*-LAKY was synthesized and purified as described previously (27). Chromatographic analysis showed no contamination of this peptide with the L-isaspartyl form. This result is supported by the expected poor recognition of this peptide by the human enzyme (27). Ovalbumin (chicken egg, Grade VII) was from Sigma. *N*-Succinyl *p*-nitroanilide peptides, succinyl derivatives, succinic acid, succinamic acid, succinamide, alanine, and alanine peptides were from Sigma. AdoHcy, 5-deoxy-5′methylthioadenosine (MTA), and adenosine were from Sigma. *N*-Succinyl-*p*-nitroanilide peptides were dissolved in 50 mM NaOH to give 10 mM solutions.

Peptide succinylation was performed using a modification of the protocol of Pearson and Kemp (34). 5 mg each of mono-, di-, tetra-, and penta-alanine and 18 mg of tri-alanine were suspended in 1 ml of 0.2 M sodium borate, pH 9. The concentration of each solution was 56, 31, 78, 16, and 13 mM respectively. To these solutions, a total of 180 μl of a 15 mg/ml solution of succinic anhydride in dimethyl formamide was added in small aliquots. The contents were constantly stirred at room temperature, and the pH was maintained at 9 with 1 M NaOH for a total incubation time of 90 min.

Methyltransferase Assay—A vapor diffusion assay was used to determine the methyltransferase activity (35). The method involves the transfer of radiolabeled methyl groups by the enzyme from *S*–adenosyl-[*methyl*-^{14}C]-L-methionine ([^{14}C]AdoMet) (57 mCi/mmol; Amersham Biosciences, Inc.) to a suitable methyl-accepting substrate. Subsequently, the methyl esters are hydrolyzed, and the resulting [^{14}C]methanol is quantified. Typically, the reaction mixture (total of 40 μl) contains 10 μM of [^{14}C]AdoMet, either a buffer of 0.33 M sodium HEPES at pH 7.5 or 0.2 M sodium citrate/phosphate at pH 4, and enzyme (0.06–0.12 μg of purified protein). In each set of assays, the activity was measured in the presence and absence of methyl-acceptor. The reaction was allowed to proceed typically at 68 °C for 1 h and was stopped by quenching with 40 μl of 0.2 N NaOH, 1% (w/v) SDS. In preliminary experiments, we demonstrated that the rate of product formation was linear with time under these conditions. The contents of the reaction mixture were vortexed, and 60 μl of this mixture was then spotted onto a 1.5 × 8-cm pleated filter paper (Bio-Rad, number 1650962), which was placed in the neck of a 20-ml scintillation vial containing 5 ml of counting fluor (Safety Solve High Flashpoint mixture; Research Products International). The vials were capped and incubated for 2 h at room temperature. During this period the resulting [^{14}C]methanol diffuses into the fluor, and the unreacted [^{14}C]AdoMet stays on the filter paper. Quantification was done by removal of the paper and counting the vials in a scintillation counter (Beckman LS 100C). Unless otherwise indicated, the specific activity was calculated by subtracting the "endogenous activity" measured in the absence of methyl-acceptor from the activity in the presence of the methyl-acceptor. This "endogenous" activity (which includes contributions from the isotope itself as well as from the presence of endogenous methyl-acceptors) was generally 2–3% of the activity with L-isoaspartyl peptide at pH 4 and 15–20% of this activity at pH 7.5.

Kinetic Analyses—In experiments where methyl-accepting substrate concentrations were varied, AdoMet was maintained at a fixed concentration of 10 μM. When AdoMet levels were varied, the peptide VYP-L-*isoAsp*-HA was used as a methyl-acceptor at a fixed concentration of 500 μM. The analyses of the human enzyme with *N*-succinyl-AAVA-*p*-nitroanilide were performed at 37 °C at pH 7.5 using 0.016 μg of purified human recombinant L-isoaspartyl methyltransferase type II (33) at a specific activity of 147 nmol/min/mg protein at 37 °C using VYP-L-*isoAsp*-HA as a methyl-acceptor. In all cases, product formation was linear with time.

Mass Spectrometry—Mass spectrometry was performed by Dr. Kym Faull at the Pasarow UCLA Mass Spectrometry Facility. HPLC fractions from the C18 reverse-phase column were collected and dried in a Speed-Vac. The dried HPLC samples were redissolved in 20 μl of water/acetonitrile/triethylamine (50:50:0.1, v/v/v), and aliquots were injected into an electrospray ionization source attached to a quadrupole mass spectrometer (Perkin-Elmer, Thornhill, Canada; Sciex API III; −3.5 Kv ion spray voltage, spray nebulization with hydrocarbon-depleted air ("zero" grade air, 40 p.s.i., 0.6 liters/min; Zero Air Generator, Peak Scientific, Chicago, IL), curtain gas (0.6 liters/min) from the vapors of liquid nitrogen, with the mass resolution set so the isotopes of the polypropylene glycol/NH_4^+ singly charged ion at *m/z* 906 were resolved with a 40% valley) scanning from *m/z* 300–2200 in the negative ion mode. The spectra were collected (step size, 0.3 Da; dwell time, 1 ms/step and 6.7 s/scan; orifice at −85 V), and the resulting spectra were summed and then corrected for the background with software supplied with the instrument.

RESULTS

Characterization of P. furiosus Recombinant L-Isoaspartyl Methyltransferase—The activity of the purified *P. furiosus* L-isoaspartyl methyltransferase was initially studied for its temperature and pH dependence. Nonthermophilic L-isoaspartyl methyltransferases display maximal activity in the range of 45–55 °C (36), whereas the enzyme from the thermophilic eubacterium *T. maritima* was found to have an optimal temperature of 85 °C (10). We first assayed the activity of the *P. furiosus* enzyme at temperatures ranging from 4 to 95 °C at pH 7.5. We found significant activity over the entire range, in-

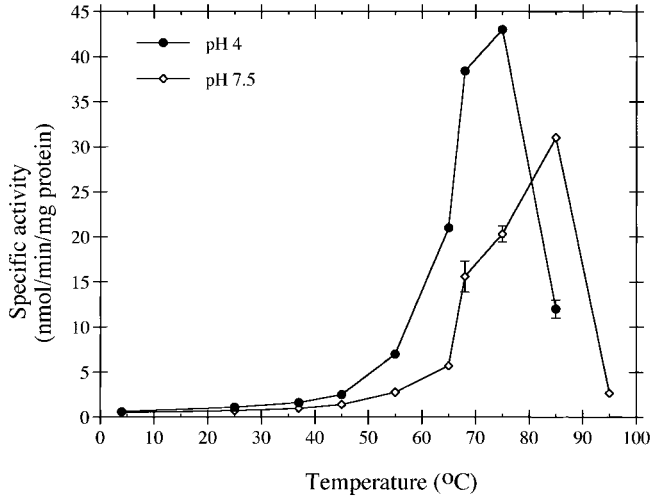

FIG. 1. **Effect of temperature on the activity of purified *P. furiosus* recombinant L-isoaspartyl methyltransferase.** The reactions were performed as described under "Experimental Procedures" using 25 μM of methyl-accepting peptide (VYP-L-*isoAsp*-HA) and 0.12 μg of enzyme. All of the reaction components were prepared in buffers of the respective pH (4 or 7.5; determined at room temperature), and incubation at each temperature was done for 20 min. The reactions were done in triplicate, and the *error bars* represent the standard deviations from the mean values. When no *error bar* is shown, the error was smaller than the *symbol*.

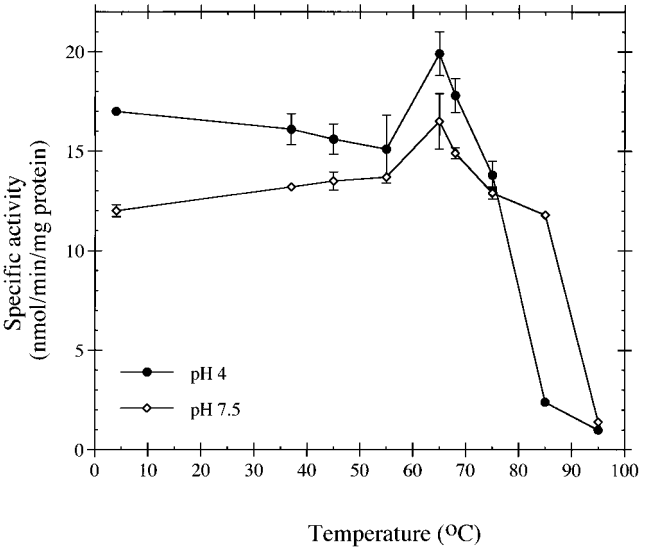

FIG. 2. **Thermal stability of the *P. furiosus* recombinant L-isoaspartyl methyltransferase.** The purified enzyme was preincubated at the indicated temperatures (4 °C-95 °C) for 60 min at either pH 4 or pH 7.5 and was then assayed for activity at 68 °C for 30 min using 25 μM of methyl-accepting peptide (VYP-L-*isoAsp*-HA) and 0.12 μg of enzyme at the same pH value as described in Fig. 1. All reactions were done in duplicate, and the values represent the means ± range. When no *error bar* is shown, the error was smaller than the *symbol*.

creasing to a maximal activity at 85 °C, with activity at 95 °C comparable with that at 55 °C (Fig. 1). To measure the thermal stability of the enzyme, the purified enzyme was preincubated at various temperatures for 1 h at pH 7.5 and then assayed at 68 °C at pH 7.5. As shown in Fig. 2, little or no loss of activity was observed up to preincubation temperatures of 85 °C, although there was an approximate 7-fold loss of activity after preincubation at 95 °C.

The enzyme was next analyzed for activity at pH values ranging from 3 to 10. Because the organism has been found to grow optimally at pH values ranging from 5 to 9 (32), we expected the enzyme to be most active in the neutral pH range. However, we found maximal activity at pH 4, dropping off to a plateau value ~4-fold lower from pH 7 to 10. The activity at pH 3 was about 25-fold lower than at pH 4 (Fig. 3). An optimal pH for L-isoaspartyl activity as low as 4 has not been previously observed for these enzymes from other organisms; maximal activity is typically found in pH values over the range of 6–8 (33, 36). The data shown in Fig. 3 suggest that a crucial amino acid in the *P. furiosus* enzyme with a pK_a of about 5 must be protonated for full activity.

We then reexamined the optimal temperature for activity and stability at pH 4. As shown in Fig. 1, the optimal temperature for the activity at pH 4 is 75 °C, about 10 °C lower than the optimal value at pH 7.5. Up to 75 °C, activity levels at pH 4 were 2–3-fold higher than those at pH 7.5. However at 85 °C, the activity at pH 4 was about 2.5-fold lower than at pH 7.5. The thermal stability of the enzyme at pH 4 was found to be similar to that observed at pH 7.5 up to 75 °C but decreased 4-fold by 85 °C, conditions where the enzyme was still stable at pH 7.5 (Fig. 2).

Sensitivity of the P. furiosus Methyltransferase to Inhibition by AdoHcy and Derivatives—Enzyme prepared for structural studies was previously found to retain tightly bound cofactors including AdoMet, AdoHcy, and surprisingly, adenosine during the course of purification and crystallization (30). AdoHcy is known to be a potent inhibitor of most methyltransferases, including the L-isoaspartyl methyltransferase from several organisms (37), whereas adenosine has not been previously reported as an inhibitor of methyltransferases. We thus examined the effect of these compounds, as well as that of another known inhibitor of this enzyme, MTA, a by-product of polyamine synthesis (38).

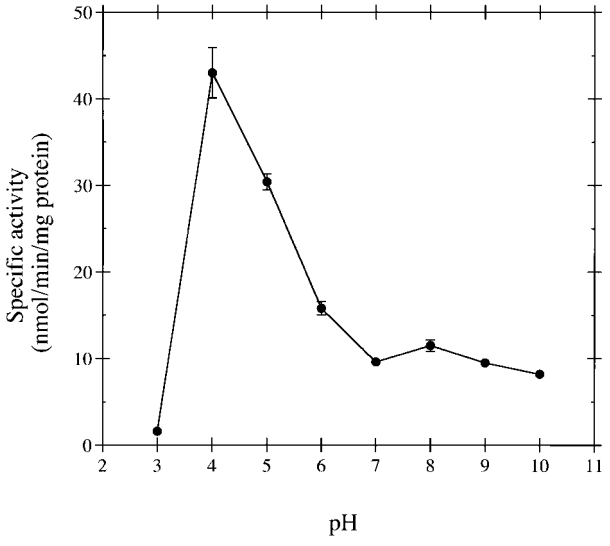

FIG. 3. **Effect of pH on the activity of purified *P. furiosus* recombinant L-isoaspartyl methyltransferase.** The activity of the purified enzyme was measured at 68 °C in final concentrations of 0.2 M disodium phosphate adjusted to pH with citric acid (pH 3–7) and 0.2 M 2-amino-2-methyl-1,3-propanediol chloride (pH 8–10) with the pH values determined at room temperature. The reactions were performed for 20 min as described under "Experimental Procedures" using 100 μM of peptide methyl-acceptor (VYP-L-*isoAsp*-HA) and 0.12 μg of enzyme. The reactions were done in triplicate, and the *error bars* represent the standard deviations from the mean values. When no *error bar* is shown, the error was smaller than the *symbol*.

In Fig. 4, we show that AdoHcy is a potent inhibitor both at pH 4 and at pH 7.5, with half-maximal inhibition in the 10 μM range. Poorer inhibition is seen with 5′-deoxy-5′-methylthioadenosine, with half-maximal inhibition at about 250 μM at both pH values. Interestingly, we did detect inhibition with

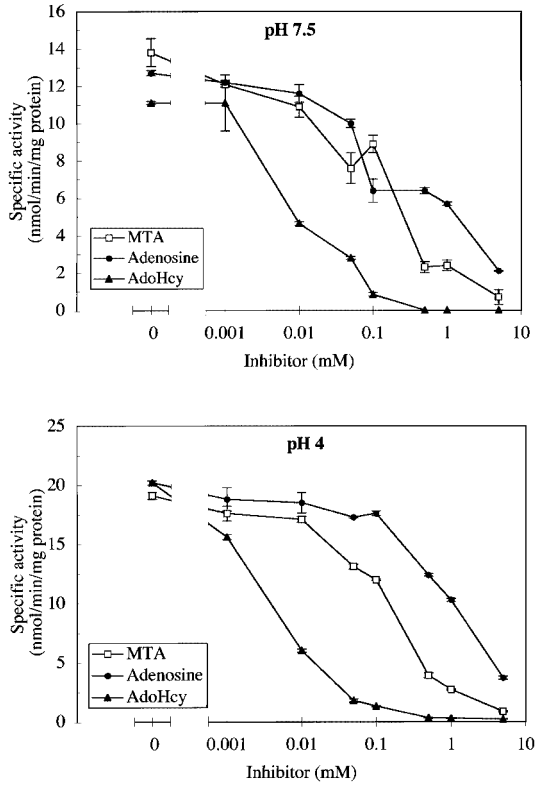

FIG. 4. **Inhibition of *P. furiosus* recombinant L-isoaspartyl methyltransferase by AdoHcy, MTA, and adenosine.** The reactions were performed as described under "Experimental Procedures" using 10 μM of peptide (VYP-L-*isoAsp*-HA) and 0.12 μg of enzyme, and the incubations were done at 68 °C for 30 min at pH 7.5 (*upper panel*) and pH 4.0 (*lower panel*). Stock solutions of AdoHcy, MTA, and adenosine were prepared in buffers of respective pH and used at concentrations ranging from 0.001 to 5 mM. All of the reactions were done in duplicate, and the values represent the means ± range. When no *error bar* is shown, the error was smaller than the *symbol*.

adenosine, with half-maximal inhibition at about 100 μM at pH 7.5 and 1 mM at pH 4.

Methyl-accepting Substrate Specificity of the P. furiosus L-Isoaspartyl Methyltransferase—We measured the methyl-accepting activity at pH 7.5 and 68 °C of the recombinant *P. furiosus* enzyme for a number of peptide and protein substrates previously characterized for other L-isoaspartyl methyltransferases (Table I). We found that the *P. furiosus* enzyme recognizes these substrates with affinities much higher than those of other prokaryotic organisms and in fact even higher in some cases than those of the human enzyme, previously determined to be the most effective catalyst of these reactions (24). The K_m values for the L-isoaspartate peptides were comparable for the *P. furiosus* and human enzymes, whereas the *P. furiosus* enzyme recognized the protein ovalbumin with about 8-fold higher affinity than the human enzyme (Table I). Remarkably, the greatest difference seen between the *P. furiosus* and the human enzyme was with respect to the methylation of a D-aspartyl-containing peptide. Here, the affinity of the *P. furiosus* enzyme was found to be about 120-fold higher (K_m = 23 μM) than that for the human enzyme (K_m = 2700 μM), whereas the maximal velocity values were similar (Table I). We also measured the affinity for the methyl donor AdoMet and found that its value was similar to that of the enzymes from other sources (Table I). These results suggest that the *P. furiosus* enzyme has evolved to be capable of recognizing both L-isoaspartate and D-aspartate residues with high affinity. A combination of direct structural analysis and molecular modeling has demonstrated that peptides containing either L-isoaspartyl or D-aspartyl residues form multiple binding contacts with the *P. furiosus* methyltransferase (30).

We then examined the enzyme kinetics at acidic pH. Although we found that the maximal activity of the enzyme was at pH 4, at this pH value the affinity for AdoMet and its methyl-accepting substrates was found to be decreased by 3–80-fold (Table I). We also examined the effect of lowering the temperature to 37 °C at pH 7.5. The K_m values measured at pH 7.5 for 37 °C were comparable with those measured at 68 °C at the same pH value for the isoaspartyl peptides but were about 7-fold higher for ovalbumin and 4-fold lower for AdoMet. These results suggest that pH has a greater effect on the substrate affinity than does the temperature for the *P. furiosus* methyltransferase. A different effect of temperature on substrate affinity has been seen with the enzyme from the eubacterial thermophile *T. maritima* (10). Here, the *Thermotoga* enzyme displayed about 8-fold higher affinities for the L-isoaspartyl peptide at 37 °C than at 85 °C.

Novel Methyl-accepting Substrates for the P. furiosus L-Isoaspartyl Methyltransferase—The observation that the *P. furiosus* L-isoaspartyl methyltransferase could efficiently methylate both L-isoaspartate and D-aspartate peptides led us to examine the minimum structural elements required within a substrate for it to be methylated. Fig. 5 (*top* and *middle panels*) illustrates the structure of isoaspartyl and aspartyl residues aligned so that the potential methyl-accepting carboxyl group is on the left separated from the carboxyl-side peptide bond on the right by two carbon atoms labeled C_A and C_B. Here, the only difference between these structures is that the α-amino group is located on C_A for an isoaspartyl residue or on the adjoining C_B carbon atom for an aspartyl residue. Because the enzyme does not apparently require the presence of the α-amino group on either C_A or C_B for catalysis, we hypothesized that the α-amino group itself might be entirely dispensable. We tested this idea by asking whether catalysis could take place on substrates containing only two hydrogen atoms on each of C_A and C_B, as would occur in *N*-succinyl peptides (Fig. 5, *bottom panel*). We thus tested a number of nonaspartyl-containing succinyl derivatives that might mimic the common features of L-isoaspartyl and D-aspartyl residues.

In initial experiments, we tested a series of commercially available *N*-succinyl-tri- and tetra-peptides containing a *p*-nitroanilide group at the C terminus (Table II). We found that all of these peptides were methylated by the *P. furiosus* enzyme despite the fact that none of them contained L-isoaspartyl or D-aspartyl residues. However, the presence of a peptide backbone fragment on the C-terminal side did appear to be essential, because no activity was seen for succinamic acid, succinic acid, or succinyl-L-homoserine, derivatives that contained at most a single peptide linkage (Table II). These results are consistent with the structure of the *P. furiosus* methyltransferase in complex with the peptide VYP-L-*isoAsp*-HA where the majority of the contacts between enzyme and substrate are seen on the C-terminal side of the peptide (30).

We then chose to examine the methylation of the *N*-succinyl-AAVA-*p*-nitroanilide peptide in more detail. In Table I, we report that the peptide is recognized with an apparent K_m value at 68 °C of 375 μM at pH 7.5 and 11 mM at pH 4. We then asked whether the human enzyme might also be able to recognize this *N*-succinyl peptide. We found that this was indeed the case and measured an apparent K_m value of 690 μM (Table I). Although these K_m values are about 500–2500-fold higher than those observed for the best L-isoaspartyl-containing peptides for L-isoaspartyl methyltransferases from other organisms, they are certainly within the range seen with a variety of more

TABLE I
Kinetic constants of P. furiosus recombinant L-isoaspartyl methyltransferase

Substrate	K_m[a]				V_{max}			
	P. furiosus			Human	*P. furiosus*			Human
	68 °C pH 7.5	68 °C pH 4	37 °C pH 7.5	37 °C pH 7.5	68 °C pH 7.5	68 °C pH 4	37 °C pH 7.5	37 °C pH 7.5
	μM				*nmol/min/mg protein*			
VYP-L-*isoAsp*-HA	0.53 ± 0	8 ± 0	0.59 ± 0.04	0.29 ± 0.03[c]	49.3 ± 0.3	164 ± 1	1.2 ± 0.03	11.4 ± 0.1
KASA-L-*isoAsp*-LAKY	0.26 ± 0.02	21 ± 2	0.55 ± 0.02	0.52 ± 0.08[c]	22.2 ± 0.4	146 ± 9	1.12 ± 0.02	7.1[d]
KASA-D-*Asp*-LAKY	23 ± 0.1	N.D.[b]	N.D.	2700 ± 400[c]	1.7 ± 0.01	N.D.	N.D.	1.7[d]
Ovalbumin	4.63 ± 0.02	166 ± 2	31.1 ± 1.4	35[c]	13.7 ± 0.1	195 ± 1	2.0 ± 0.04	7.9[d]
AdoMet	3.41 ± 0.01	10 ± 0	0.95 ± 0.07	2.2 ± 0.2[c]	47.9 ± 0.01	351 ± 1	1.7 ± 0.03	N.D.
N-Succinyl-AAVA-*p*-nitroanilide	375 ± 40	11,000 ± 2,000	N.D.	690	10.8 ± 0.14	107 ± 2	N.D.	2.0 ± 0

[a] K_m values were calculated by fitting substrate concentration/velocity data to the Michaelis-Menten equation using Biomechanic software obtained at www.biomechanic.org. The values represent the means ± S.D.
[b] N.D., not done.
[c] The results are from Lowenson and Clarke (27) for reactions done at pH 7.4.
[d] The values were calculated from the relative V_{max} values in Ref. 27 based on the value obtained here for the VYP-L-*isoAsp*-HA peptide.

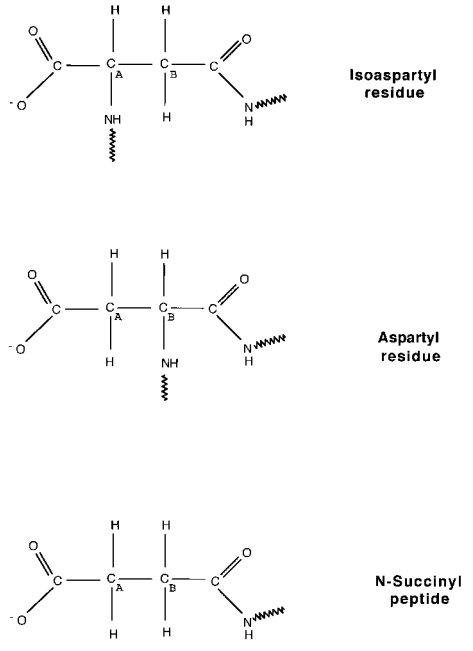

FIG. 5. **Structures of methyl-accepting substrates of the L-isoaspartyl (D-aspartyl) methyltransferase.** Isoaspartyl (*top*) and aspartyl (*middle*) residues are drawn with the carboxyl group that becomes methyl esterified in the L-isoaspartyl and D-aspartyl configurations to the *left* and the carboxyl-side peptide bond on the *right*. The common feature of these two residues is an *N*-succinyl peptide as shown at the *bottom*.

poorly recognized L-isoaspartyl-containing peptide substrates for the human enzyme (25).

In these initial experiments, the *N*-succinylated peptides were dissolved in an alkaline solution. We then asked whether the peptide might be partially hydrolyzed under these conditions to release *p*-nitroanilide. HPLC and mass spectral analyses of the solution revealed the presence of not only *N*-succinyl-AAVA-*p*-nitroanilide as expected but also the hydrolysis products *N*-succinyl-AAVA and *p*-nitroaniline (Fig. 6). We found that both the *p*-nitroanilide and free carboxyl forms of the *N*-succinyl-AAVA peptide were good methyl-acceptors for the *P. furiosus* enzyme (Fig. 6).

We then focused on understanding the relationship between the number of amino acid residues of the *N*-succinylated peptide, reflecting the length of the peptide on the C-terminal side of an L-isoaspartyl or D-aspartyl peptide and their ability to be methyl esterified by the *P. furiosus* enzyme. We thus chemically succinylated mono-, di-, tri-, tetra-, and penta-alanines with succinic anhydride and then tested each reaction mixture as a source of methyl-acceptors (Fig. 7). Under these conditions, we found that the tetraalanine derivative was the best methyl acceptor, with reduced activity toward the trialanine and pentaalanine derivatives and little or no activity with the alanine or the alanine dipeptide. In control experiments, we found no methyl esterification when succinic anhydride was deleted from the reaction mixture (data not shown).

Finally, to demonstrate that the presence of other components in the succinylation reaction mixtures or variable reaction yields did not skew the results of the experiments described above, we purified the *N*-succinyl-tri-, tetra-, and penta-alanine peptides by HPLC. As shown in Fig. 8, we were able to separate each of these species and confirm their identity by electrospray mass spectrometry. We found here that *N*-succinyl-tetraalanine was the best substrate, with less but still significant activity with the tri- and penta-derivatives, confirming the results shown in Fig. 7.

Given the ability of the *N*-succinyl group to mimic L-isoaspartyl and D-aspartyl residues, how is it possible that the enzyme does not recognize L-aspartyl groups as well? From the three-dimensional structure determined for the *P. furiosus* enzyme in complex with VYP-L-*isoAsp*-HA and modeling studies with the corresponding D-aspartyl peptide (30), it appears that although the substrate α-amino group is not essential for binding, it can effectively block the binding of substrates that contain L-aspartyl or D-isoaspartyl residues. Additionally, the configuration of the D-aspartyl peptide required for enzyme binding is not possible in the L-aspartyl peptide because of steric overlaps (30).

It is interesting to note that the α-amino group is also not required for recognition of peptides by the isoprenylcysteine methyltransferase, where efficient catalysis occurs with *S*-farnesylthiopropionate, a molecule devoid of any nitrogen atom (39). For the *P. furiosus* L-isoaspartyl methyltransferase, there is clearly an enhancement of methyl-accepting activity with the inclusion of amino acid residues on the N-terminal side of the L-isoaspartyl or D-aspartyl residues (Table I). However, at least for the peptide VYP-L-*isoAsp*-HA in complex with the methyltransferase, the majority of methyl-acceptor-enzyme interactions seen are with the backbone of the C-terminal histidine and alanine residues rather than the N-terminal valine, tyrosine, and proline residues (30). Studies with the human enzyme have also suggested that at least the two residues following the methyl-accepting residue are crucial for maximal binding efficiency (25).

DISCUSSION

The L-isoaspartyl/D-aspartyl methyltransferase is an unusual enzyme in that it can catalyze the methyl esterification of both isomerized L-aspartyl residues and racemized D-aspartyl

TABLE II
Activity of P. furiosus recombinant L-isoaspartyl methyltransferase toward succinyl compounds

Substrate[a]	Activity[b]	Activity relative to isoaspartyl peptide[c]
	nmol/min/mg protein	%
None (endogenous)	3.8 ± 0.2	0
Succinyl-AAA-*p*-nitroanilide	9.4 ± 0.2	31
Succinyl-AAV-*p*-nitroanilide	15.8 ± 0.3	67
Succinyl-AAVA-*p*-nitroanilide	17.8 ± 0.2	79
Succinyl-AAPF-*p*-nitroanilide	11.8 ± 0.3	45
Succinyl-AAPL-*p*-nitroanilide	10.2 ± 0.1	36
O-Succinyl-L-homoserine	3.5 ± 0.1	−2
Succinyl choline chloride	2.8 ± 0.1	−6
Succinyl acetone	3.5 ± 0.1	−2
Succinic acid	2.9 ± 0.3	−5
Succinamic acid	3.7 ± 0.1	0
Succinamide	3.9 ± 0.2	1
VYP-L-*isoAsp*-HA	21.6 ± 0.2	100

[a] Substrates were used at a final concentration of 1 mM.
[b] Reactions were performed at 68 °C at pH 7.5. Activity (mean ± range of duplicate assays) was determined from the total radioactivity found as methyl esters; no background was subtracted.
[c] The endogenous activity (no substrate) was subtracted as a background.

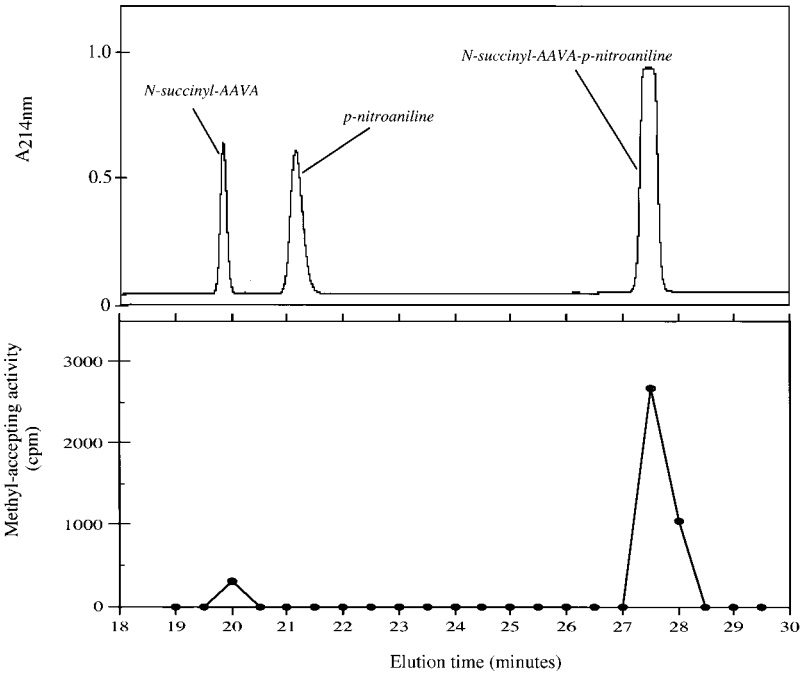

FIG. 6. **Methyl-acceptor analysis of *N*-succinyl-AAVA-*p*-nitroanilide and its hydrolysis products.** *N*-Succinyl-AAVA-*p*-nitroanilide was chromatographed using reverse-phase HPLC where 20 μl of a 10 mM solution dissolved in 50 mM NaOH was injected into an Econosphere reverse-phase C18 column (5-μm spherical beads, 4.6-mm inner diameter × 250-mm length). The column was equilibrated with solvent A (0.1% trifluoroacetic acid in water) and eluted using a linear gradient from 100% solvent A to 100% solvent B (0.1% trifluoroacetic acid, 90% acetonitrile, 9.9% water) over a 45-min period at a flow rate of 1 ml/min. 0.5-ml fractions were collected and monitored by the absorbance at 214 nm (*upper panel*). Three UV-absorbing peaks were found at 20, 21.5, and 27.5 min. The peaks at 20 and 27.5 min were identified by negative ion mode electrospray mass spectroscopy to be *N*-succinyl-AAVA (*m/z* 429.3, expected 429.3) and *N*-succinyl-AAVA-*p*-nitroanilide (*m/z* 549.1, expected 549.3), respectively. The material at 21.5 min was identified as *p*-nitroaniline by its yellow color and comigration with a synthetic standard. Fractions between 19 and 30 min were dried in a vacuum centrifuge and resuspended in 50 μl of water, and 20 μl of each fraction was assayed for its ability to be methylated by 0.12 μg of the purified *P. furiosus* enzyme with 10 μM of [^{14}C]AdoMet and incubation at 68 °C for 1 h at pH 7.5 in duplicate. Methyl-accepting activity is shown as base-labile, volatile radioactivity in the *lower panel*.

residues while not recognizing either the normal L-aspartyl or the racemized and isomerized D-isoaspartyl derivative (40). The ability of this enzyme to methylate abnormal aspartyl residues in polypeptides was first described as an activity on D-aspartyl residues in human erythrocyte membrane proteins and led to the idea that this enzyme might recognize spontaneously damaged proteins for repair (26). Subsequently, it was found that the enzyme would also catalyze the methylation of L-isoaspartyl residues in peptides (40, 41). The physiological importance of enzymatic D-aspartyl methylation was then questioned when it was found that the affinity of the human enzyme for D-aspartyl-containing peptides was 700–10,000 times lower than that for corresponding peptides containing L-isoaspartyl residues (27). Additionally, no activity on D-aspartyl residues in short peptides was found with L-isoaspartyl methyltransferases isolated from *E. coli* (28), *T. maritima* (10), *Arabidopsis* (24), and nematodes (13), suggesting that the ability to methylate D-aspartyl residues might be a special adaptation of the methyltransferases in complex and long-lived mammalian species.

Our finding here that the *P. furiosus* enzyme recognizes D-aspartyl residues in peptides with a 120-fold higher affinity than the human enzyme suggests, however, that the ability of cells to recognize spontaneously damaged proteins containing

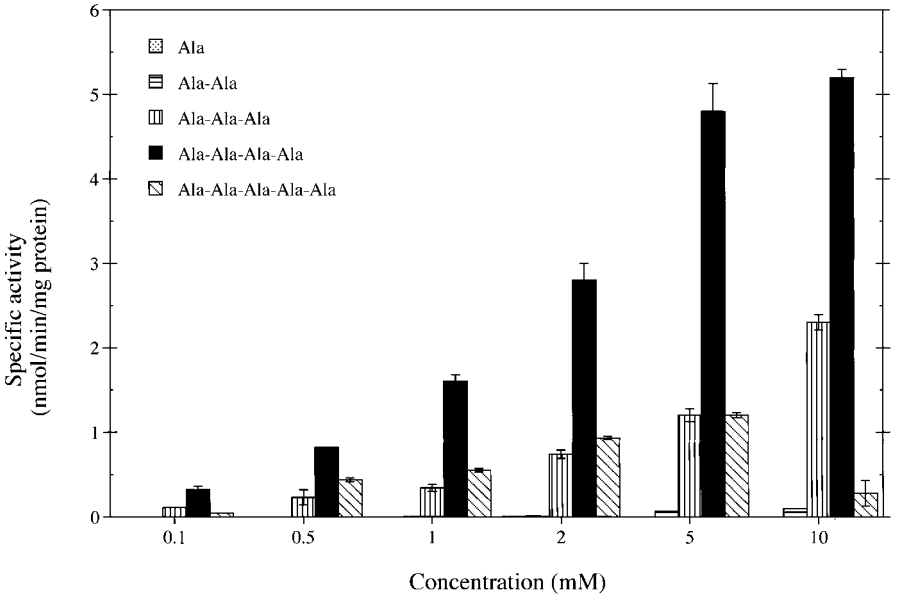

FIG. 7. **Methylation of chemically succinylated alanine peptides.** Alanine peptides were succinylated as described under "Experimental Procedures." Aliquots of the total 1-ml reaction mixture were diluted suitably to give a final concentration ranging from 0.1 to 10 mM in a 40 μl reaction volume and assayed at pH 7.5 using 10 μM of [^{14}C]AdoMet and 0.12 μg of purified *P. furiosus* enzyme. The reactions were incubated at 68 °C for 1 h. All reactions were done in duplicate, and the values represent the means ± range. When no *error bar* is shown, the error was smaller than the *symbol*.

D-aspartyl residues may be even more important in cells subjected to environmental conditions where spontaneous racemization reactions would be expected to be enhanced. When normal L-aspartyl and L-asparaginyl residues spontaneously degrade, the major product is the L-isoaspartyl residue. Methyltransferase-initiated conversion of these residues back to L-aspartyl residues can thus reverse the bulk of the damage. However, D-aspartyl and D-isoaspartyl residues can also accumulate by the facile racemization of the succinimide intermediate (1, 42). Methylation of D-aspartyl residues can also lead to their eventual conversion to normal L-aspartyl residues (27), but D-isoaspartyl residues would be expected to be unaffected or even to increase as a result of an increased steady-state level of D-succinimide residues. Interestingly, analysis of aged lens proteins demonstrated the presence of mainly L-aspartyl and D-isoaspartyl residues (43); the relative scarcity of L-isoaspartyl and D-aspartyl residues can be attributed to the repair action of the methyltransferase. It is unclear, however, why one thermophilic organism (*P. furiosus*) would have an enzyme designed to efficiently recognize D-aspartyl residues while another (*T. maritima*) would not.

Analysis of the structural features that could lead to the recognition of D-aspartyl and L-isoaspartyl residues but not normal L-aspartyl residues suggested that the presence of the peptide amino grouping on the N-terminal side of the residue plays only a secondary role in enzymatic recognition. We found here that *N*-succinyl-peptides, which totally lack the amino group and structurally resemble both D-aspartyl and L-isoaspartyl residues, are good methyl-accepting substrates for the *P. furiosus* and the human methyltransferase. We have considered the possibility that these enzymes may normally recognize *N*-succinyl peptides *in vivo*. *N*-Succinyl polypeptides may form via the nonenzymatic reactivity of the nucleophilic peptidyl α-amino groups on electrophilic molecules such as succinyl CoA. Succinylation of amino groups can produce a large conformational change in some proteins (44) and none in others (45). It will be interesting to see whether these modified proteins may in fact be recognized by the methyltransferase and how the methylated protein may be metabolized.

The spontaneous generation of L-isoaspartyl and D-aspartyl residues can result in a loss of protein function, both in protein pharmaceuticals (46) as well as in organisms (9, 21). The mammalian L-isoaspartyl methyltransferase has been useful in detecting such damage (47). However, our characterization of the

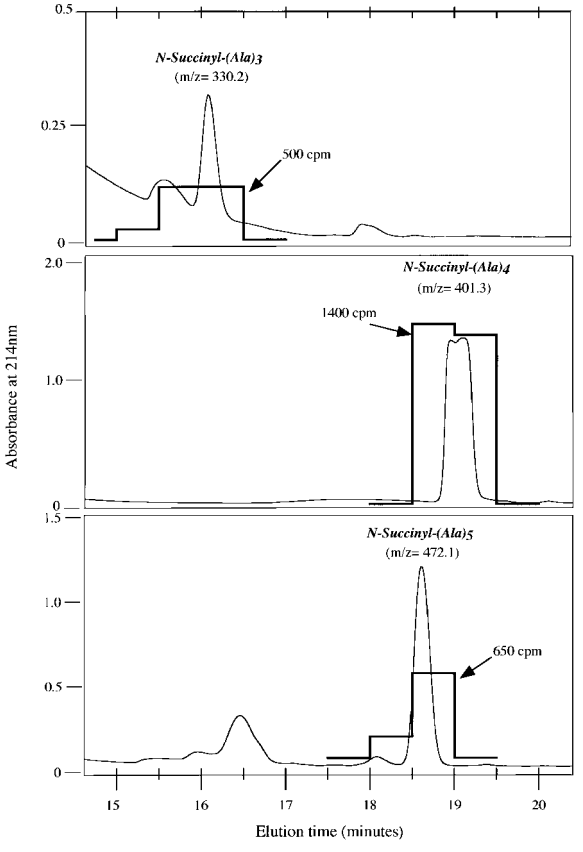

FIG. 8. **Methyl-acceptor activity of purified *N*-succinyl-tri-, tetra-, and penta-alanine.** The products of succinylation reaction mixtures of alanine peptides (25 μl) were fractionated by HPLC as described in Fig. 6, with peak monitoring by absorbance at 214 nm. The *upper panel* shows the reaction products with trialanine, the *middle panel* shows the reaction products with tetraalanine, and the *lower panel* shows the reaction products with pentaalanine. *N*-Succinyl-peptide products were identified by electrospray mass spectrometry in negative ion mode as described under "Experimental Procedures," and the *m/z* ratios are indicated. Fractions eluting between 15 and 20 min were dried and dissolved in 30 μl of water, and 15 μl of each was assayed for methyl-accepting activity at pH 7.5, 68 °C, for 1 h using 10 μM of [^{14}C]AdoMet and 0.12 μg of purified *P. furiosus* methyltransferase. Methyl-accepting activity is shown by the *bold bar lines* superimposed on the chromatogram, with the maximal radioactivity indicated.

P. furiosus enzyme suggests that this species may be a superior analytical reagent for this purpose because it can recognize L-isoaspartyl peptides with an affinity similar to that of the human enzyme, whereas it can recognize protein substrates such as damaged ovalbumin even better than the human enzyme and importantly can recognize D-aspartyl-containing peptides with much higher affinity.

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