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Functional properties of the thermostable mutL from *Thermotoga maritima*

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The methyl-directed mismatch repair (MMR) mechanism has been extensively studied *in vitro* and *in vivo*, but one of the difficulties in determining the biological relationships between the MMR-related proteins is the tendency of MutL to self-aggregate. The properties of a stable MutL homologue were investigated using a thermostable MutL (TmL) from *Thermotoga maritima* MSB8 and whose size exclusion chromatographic and crosslinking analyses were compatible with a dimeric form of TmL. TmL underwent conformational changes in the presence of nucleotides and single-stranded DNA (ssDNA) with ATP binding not requiring ssDNA binding activity of TmL, while ADPnP-stimulated TmL showed a high ssDNA binding affinity. Finally, TmL interacted with the *T. maritima* MutS (TmS), increasing the affinity of TmS to mismatched DNA base pairs and suggesting that the role of TmL in the formation of a mismatched DNA-TmS complex may be a pivotal observation for the study of the initial MMR system. [BMB reports 2009; 42(1): 53-58]

INTRODUCTION

In living organisms methyl-directed mismatch repair (MMR) is the primary process for correcting replication errors and unfavorable recombinations (1,2). The essential components of the prokaryotic MMR system are MutS, MutL, MutH, UvrD, several exonucleases (ExoI/VII/X), RecJ, DNA polymerase III, single-stranded DNA binding protein, and DNA ligase (3-5). The inactivation of these proteins affects the information integrity of the chromosomes, genomic stability, and the intracellular mutation frequency (6).

As a mediator in the MMR pathway, MutL possesses a relatively weak ATP hydrolytic activity, compared to other ATPase proteins (7), as well as a nonspecific DNA binding activity, especially to single-stranded DNA (ssDNA) (8-10). X-ray crystallographic data suggests that MutL ATP binding and hydrolysis causes major conformational changes which affect interactions with other MMR-related proteins (11, 12). Since the inclusion of ssDNA can stimulate MutL-catalyzed ATP hydrolysis, many studies have examined the correlation between the DNA binding properties of MutL and its ATPase activity (8, 10).

In spite of many efforts to clarify the functional role of MutL in the MMR system, the biological functions of MutL and its homologues remain unclear. The self-aggregation of MutL in solution poses difficulties in the study of the functional details of MMR-related proteins. In this study, a thermostable MutL protein, termed TmL, from the fully sequenced hyperthermophilic bacterium *Thermotoga maritima* MSB8 was characterized and it was shown that nonaggregated TmL retained its ATP hydrolysis capability and ssDNA binding activities at high temperatures. The formation of the TmL-TmS complex on mismatched DNA affected the binding affinity of added nucleotides for the mismatched DNA. These observations of the biological function of TmL were used to study the protein-protein interactions among MMR-related proteins and to further define the MMR mechanism.

RESULTS AND DISCUSSION

The thermostable TmL was revealed to dimerize by size exclusion chromatographic and crosslinking analyses

The functional activities of TmL, TmL, and its mutants (E35A, R101F, R261H, N300A, and K305A) were examined by preparation with chromatographic methods, as described in the Materials and Methods, and included a heat inactivation step during purification to remove the endogenous proteins that were extracted from the cultured BL21 Star™ (DE3) cells. The comparative thermostabilities and native molecular weights of TmL and *E. coli* MutL were determined by size exclusion chromatography (SEC) utilizing a Superdex 200 HR chromatography system calibrated with standard proteins. In a previous report, elution of *E. coli* MutL from a Superdex 200 HR gel filtration column showed two peaks, a major peak of a complex greater than 200 kDa and a minor peak of a complex of less than 200 kDa (11), indicating the presence of the aggregated formation of *E. coli* MutL. While the *E. coli* MutL displayed both the aggregated peak (at 8.3 mL elution volume) and the native peak (at 11.5 mL), the TmL showed only one peak (at 12.2 mL) of 110-130 kDa (Suppl. Fig. 1), but the dimeric TmL

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did not show an aggregated form.

TmL displayed nucleotide- and ssDNA-dependent conformational changes

Homodimeric interactions at the N-terminal region of *E. coli* MutL has been previously shown and single-cysteine *E. coli* MutL variants found to be crosslinked with the bifunctional linkers bis-maleimidoethane and 1,11-bis-maleimidotetraethyleneglycol, both in the presence and absence of nucleotides (13). A crosslinking assay of the *E. coli* MutL and its N-terminal domain showed that incubation with ADPnP affects its conformational changes and multimerization (11). The present results revealed that the treatment of TmL with BS^3 produced a crosslinked dimeric form of native TmL, with sizes of ~120 kDa and >220 kDa (Fig. 1A, lanes 3-5). Furthermore, addition of ADP and ADPnP yielded only a dimeric form of TmL. Unlike *E. coli* MutL, the addition of 1 mM ADP or ADPnP led to decreased yields of crosslinked TmL compared to TmL apoenzyme (apo-TmL) , suggesting that adding nucleotides to TmL resulted in a compacted TmL structure and that the closed formation of a compacted TmL may have interfered with BS_3 reactivity. The overall conclusion here was that nucleotide binding may specifically induce conformational changes in TmL.

Conformational changes were examined further by following the time course of a chymotryptic digestion of TmL in the presence of 1 mM ADP and 1 mM ADPnP. TmL was dissolved into approximately 52% non-proteolytic TmL (Fig. 1B). One 40 kDa and two 20 kDa fragments were detected from the dissolved TmL-ADP and -ADPnP complexes. Adding 1 μM ssDNA resulted in a 64% digestion and, in contrast, only 5% of the TmL was digested in the presence of 1 mM novobiocin (yields summarized in Fig. 1B), suggesting that the relative differences in the percentages of digested TmL indicated conformational changes due to interactions with ssDNA, dsDNA, ADPnP, or novobiocin (Suppl. Figs. 2C and 2D). The TmL-ADP and -ADPnP complexes were cleaved less than was the TmL-ssDNA complex. At 30 min, adding ssDNA and dsDNA to the TmL-ADPnP complex decreased the yield of cleaved TmL, while at 90 min, DNA additions to the TmL-ADPnP complex had no effect. Thus, these results agreed with the results of the crosslinking assay and, additionally, the significantly decreased yield of cleaved TmL observed with the addition of novobiocin suggested that novobiocin might have bound to TmL.

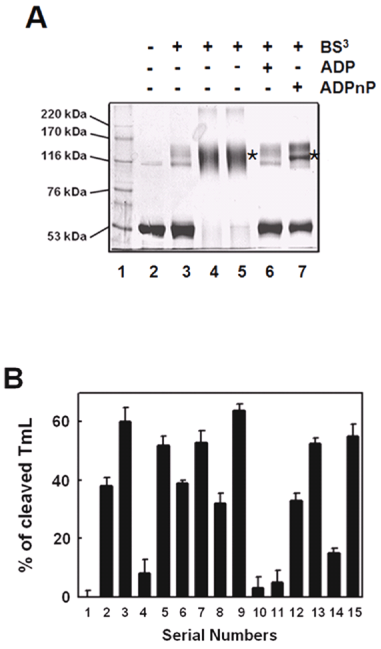

Fig. 1. Chemical crosslinking and limited digestion analyses. (A) TmL (10 μM) was reacted with BS^3: lane 1, high molecular weight marker; 2, TmL before treatment with BS^3; 3-5, TmL treated with 0.1, 0.4, and 1 mM BS^3, respectively; and 6-7, lane 5 conditions with 1 mM ADP and ADPnP, respectively. Asterisk shows dimeric TmL. (B) Percentage yields of protease-digested TmL from SDS-PAGE in Supplementary Figures 2A-D: lanes 1-3, TmL digestions at 0, 30, and 90 min after chymotrypsin treatment, respectively; 4-5, 6-7, 8-9, and 10-11, percentage yield of digested TmL with ADP, ADPnP, ssDNA, and novobiocin, respectively, at 30 (even lanes) and 90 min (odd lanes); and 12-13 and 14-15, conditions of lanes 6 and 7 with addition of ssDNA and dsDNA, respectively.

TmL and its mutants had ssDNA binding properties

Previous studies have indicated that MutL binds to ssDNA with nucleotide correlation (9, 10). *E. coli* MutL can bind to a 93 bp partial duplex DNA with an apparent K_D of 25 and 2.8 nM in the absence and presence of ADPnP, respectively (10). The investigation of the DNA binding affinity of TmL by a double-filter method performed using 30-mer ssDNA and dsDNA showed the μM range of Kd for TmL binding to a 30 bp dsDNA in the absence or presence of ADPnP. However, in the absence of ADPnP, TmL bound to ssDNA with an apparent K_D value of 53.75 ± 5.32 nM and, in the presence of ADPnP, exhibited a higher K_D of 25.65 ± 8.47 nM, with a relatively high error-range. These K_D values were larger than those form previous *E. coli* MutL studies (10), but the present results were consistent with previously reported data regarding the ability of bacterial MutL to bind ssDNA in the presence of nucleotides (10, 12, 13).

E. coli MutL mutants (E29A, R95F, R261H, N302A, and 307A) are forms which lack ATPase activity (12). The effect of ssDNA binding activity on ATP hydrolysis-related residues by sequence alignment between TmL and *E. coli* MutL (Suppl. Fig. 3) was investigated by analyzing the ssDNA binding activities of apo-TmL and its mutants (E35A, R101F, R261H, N300A, and K305A) using a gel retardation assay. The ssDNA binding activities of TmL at 55°C were found to be higher than that at 37°C (Fig. 2A) and TmL in the presence of ADP or ATP exhibited decreased ssDNA binding, the latter observation suggesting that the inclusion of nucleotides interfered with the formation of TmL-ssDNA complexes. Confirmation of ssDNA binding activity in a non-hydrolytic ATP reaction was achieved by addition

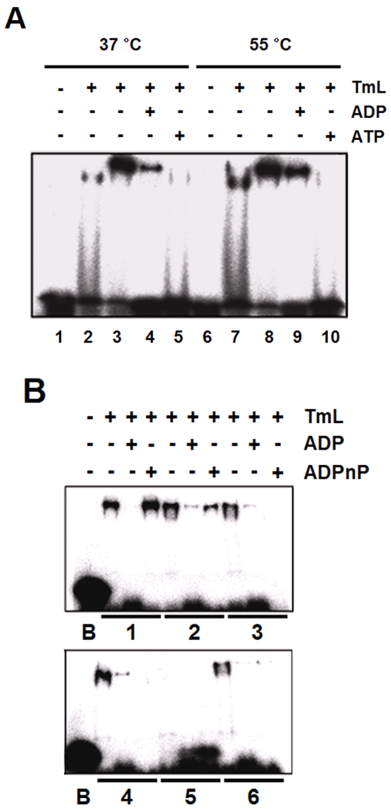

Fig. 2. Gel retardation assay for TmL-ssDNA binding in the presence of ATP, ADP, and ADPnP. (A) Lanes 1-10, the effects of ADP and ATP on TmL and 30-mer ssDNA (10 nM) interaction at 37°C and 55°C: lanes 1-3 and 6-8, 0, 0.05, and 0.2 μM TmL, respectively, and 4, 5, 9, and 10, 0.2 μM TmL treated with indicated nucleotides. (B) All lanes, except lane B in the absence of TmL, show the effect of ADP and ADPnP on the interaction of ssDNA and TmL (lane 1) or its mutants, E35A (2), R101F (3), R261H (4), N300A (5), and K305A (6).

of 1 mM ADPnP (Fig. 2B), which increased the formation of a TmL-ssDNA complex, whereas the ssDNA binding activity of the E35A-ADPnP complex was decreased, and the ssDNA binding bands of R101F-, R261H-, N300A-, and K305A-ADPnP complexes were small or not detected. This suggested that the TmL-ssDNA complex may be more functional than the apo-TmL protein, especially for non-hydrolytic ATP binding, and lead to the conclusion that the function of TmL in binding DNA with nucleotides may have influenced its structural transition and its relationship with other MMR proteins.

TmL showed ATP hydrolytic activity on ssDNA

In the absence or presence of 1 μM ssDNA, the ATPase activity of *E. coli* MutL, determined using a fluorimetric method, had k_{cat} values of ~0.13 and 0.19 min^{-1} and K_m values of ~86.62 and 40.44 μM at 37°C, respectively (Table 1).

Table 1. Kinetic data for ATP hydrolysis of TmL at 37°C

	K_m (μM)	K_{cat} (min^{-1})	K_{cat}/K_m ($\mu M^{-1}min^{-1}$)
E. coli MutL	86.62	0.13	0.0015
E. coli $MutL_{SD}$*	40.44	0.19	0.0047
T. maritima MutL	176.14	0.42	0.0024
T. maritima $MutL_{SD}$*	84.46	0.63	0.0074

*$MutL_{SD}$ indicates MutL in the presence of 30-mer ssDNA

Similarly, in the absence or presence of 1 μM ssDNA, TmL hydrolysis of ATP had k_{cat} values of ~0.42 and 0.63 min^{-1} and K_m of ~176.14 and 84.46 μM at 37°C, respectively (Suppl. Fig. 4B). Thus, the ATP hydrolytic activity of TmL appeared ~3.5 times faster than *E. coli* MutL under either condition, which suggested that TmL hydrolyzed ATP faster than *E. coli* MutL. In addition, the ATP hydrolytic activity of TmL was 1.5-fold higher in the presence of the 30-mer ssDNA than in the absence of DNA. The ATP hydrolytic activity of TmL was monitored for 90 min at 37°C and 55°C, both in the absence and presence of ssDNA, using PEI-TLC and the plot of the results indicated that the ATP hydrolysis of TmL at 37°C was higher in the presence of ssDNA than in its absence (Suppl. Fig. 4C). These results were similar to the fluorimetric data obtained in the absence and presence of ssDNA, but at 55°C the ATP hydrolytic activity of TmL was similar in both the absence and presence of ssDNA (Suppl. Fig. 4D), from which it was possible to draw the conclusion that the similarity in TmL ATPase activity levels at 55°C was due to weakened interactions between the TmL and ssDNA.

The possible intrinsic ATPase activity of TmL was examined by the preparation of TmL mutants (E35A, R101F, R261H, N300A, and K305A) with amino acid residues changed from the aligned amino acid sequences of TmL and *E. coli* MutL and PEI-TLC analysis of their ATP hydrolytic activities. The results revealed that TmL and its mutants had higher activities at 55°C than at 37°C (Suppl. Figs. 5A and 5B), indicating the thermal preference of these thermostable proteins, and all mutants unexpectedly showed less defective ATP hydrolysis. These results indicated that TmL had different structural alterations during ATP binding and hydrolysis than was previously reported for *E. coli* MutL (8). Therefore, it was concluded here that TmL was an authentic ATPase protein.

TmS bound itself to mismatched DNA in the presence of TmL and nucleotides

TmL interactions with the His-tagged TmS-based Ni-NTA affinity column were detected by absorbance at 595 nm using a Bradford assay (14) and concentrated fractions (a number of fractions from 18 to 32) analyzed by SDS-PAGE (Suppl. Fig. 6). The effect of TmL on TmS was demonstrated by examination of the mismatched DNA binding activity of TmS in the presence of TmL and nucleotides using an electrophoretic mobility

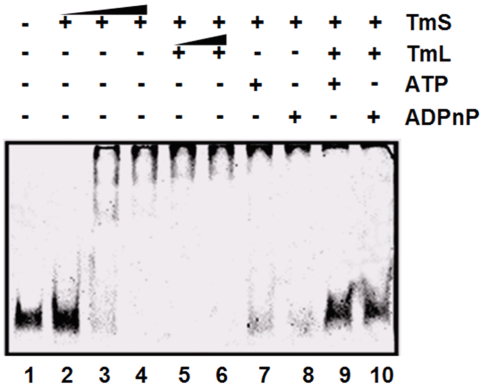

Fig. 3. Effects of TmL on the mismatched DNA binding activity of TmS by gel retardation assay. Effects of 1 mM ATP and ADPnP on the interaction between TmL (1 and 2 μM), TmS (0, 1, 2, and 4 μM) and the mismatched DNA (1 μM) shown by gel retardation assay.

shift assay (EMSA). Mismatched DNA substrate was prepared by annealing two oligonucleotides, described above, resulting in G:T mispaired dsDNA in the central region of the substrate. TmS bound to the mismatched DNA in a concentration-dependent manner (Fig. 3, lanes 2-4) and additions of TmL into the TmS-mismatched DNA complex under the same conditions increased the mismatched DNA binding activity (Fig. 3, lanes 5 and 6). Shifted bands were also observed in the upper middle region, with the addition of increasing concentrations of TmS or TmL causing a gradual, increasing shift. The addition of either ATP or ADPnP during the formation of the TmL-TmS complex on the mismatched DNA decreased the binding affinity for the DNA (Fig. 3, lanes 7-10). The DNA binding activity of TmS on the DNA could be estimated from the decrease of free DNA (unbound DNA to TmS) with the addition of TmL and nucleotides which showed that TmL significantly retarded the ATP-dependent activity of TmS on mismatched DNA. In addition, the interaction of TmL with TmS required that TmS be in an ATP-induced DNA binding mode, demonstrating that the TmL and TmS binding activities were similar to those of *E. coli* MutL and MutS (15). This suggested that the effect of TmL on the formation of a mismatched DNA-TmS complex may have important implications for the initial MMR system.

MATERIALS AND METHODS

Protein preparations

The mutL and *mutS* genes were amplified from *T. maritima* MSB8 genomic DNA by PCR using gene specific primers (*mut*L-F: 5'-CGCGGATCCGTGGAGAGGTGTTCTGTTTT-3', *mut*L-R: 5'-CCCAAGCTTTTAACGCTCGAAAAATCGGTC-3', *mut*S-F: 5'-CGGGATCCGTGAAGGTAACTCCCCTCAT-3', *mut*S-R: 5'-CGAAGCTTTCAAACAGGAAAGAGAGGAATTTG-3'). TmL was overexpressed in the BL21 Star™ (DE3) (Invitrogen, USA) strain using pET-28aTEV (Tobacco etch virus), containing the TEV protease recognition site and TmL mutants (E35A, R101F, R261H, N300A, and K305A) prepared by site-directed mutagenesis. Supernatant from the lysed cell mixture was applied to a nickel-nitrilotriacetic acid (Ni-NTA, GE Healthcare, Denmark) affinity column equilibrated in lysis buffer (20 mM Tris-HCl, pH 8.0, 500 mM NaCl, 0.5 mM β-mercaptoethanol, 5% glycerol, and 5 mM imidazole). Solutions of the cleaved TmL and its mutants were reloaded onto the Ni-NTA column, then loaded onto a Mono Q column (GE Healthcare, Denmark), equilibrated in buffer I (50 mM Tris-HCl, pH 9.0, 125 mM NaCl, 1 mM dithiothreitol (DTT), and 1 mM ethylenediaminetetraacetic acid (EDTA)), and eluted in buffer I with a linear gradient of 0.5 M NaCl. TmL and its mutants were finally purified using a Superdex 200 HR gel filtration column (GE Healthcare, Denmark) and equilibrated with buffer II (20 mM Tris-HCl, pH 8.0, 200 mM NaCl, 1 mM DTT, 1 mM EDTA, and 5% glycerol). *E. coli* MutL was purified according to previously reported procedures (11, 12) and purified proteins concentrated using an Ultracel Amicon YM-10 (Millipore, USA).

The thermostabilities and molecular sizes of the native TmL and *E. coli* MutL proteins were determined by preincubation at 37°C for 1 hr and chromatography on a Superdex 200 HR column calibrated using standard proteins (bovine serum albumin (132 and 66 kDa, dimeric and monomeric forms, respectively) and lysozyme (14 kDa)) in buffer II.

Protein cross-linking analysis

Crosslinking was assayed using a final concentration of 1 mM bis(sulfosuccinimidyl) suberate (BS_3, Sigma, USA) (11). Reaction mixtures (15 μL) containing reaction buffer I, 1 mM nucleotide (ADP or ADPnP), 1 mM novobiocin (a competitive inhibitor of the GyrB ATPase), 1 μM 30-mer ssDNA, and 10 μM TmL were incubated at 37°C for 30 minutes, the reactions terminated by addition of 0.1% bromophenol blue, 50% glycerol, 1 M Tris-HCl (pH 7.0), and 14.4 mM β-mercaptoethanol, and then analyzed by SDS-PAGE.

Limited proteolytic digestion analysis

Limited chymotryptic digestion was carried out in buffer II at 37°C with the protease-to-TmL ratio adjusted to 1:1,000 (wt/wt) for time-resolved digestion with approximately 2.5 μg of initial TmL. Sixteen μL aliquots of the reaction mixture were withdrawn at specific time intervals, combined with 4 μL of the electrophoretic loading buffer (60 mM Tris-HCl (pH 6.8), 25% glycerol, 2% SDS, 14.4 mM β-mercaptoethanol, and 0.1% bromophenol blue), immediately heated in a water bath at 37°C for 10 minutes to quench proteolysis, and subjected to SDS-PAGE analysis. Various TmL digestion patterns were observed by addition of 1 μM ssDNA, 1 mM nucleotides (ADP or ADPnP), and 1 mM novobiocin to the proteolytic reaction mixtures. The percentage (%) of cleaved TmL was calculated by dividing the intensity of the digested TmL band (band at ~60 kDa) by the intensity of the undigested TmL band (band at ~60 kDa) times 100%.

ATPase activity assay

The ATPase activities of TmL and its mutants (E35A, R101F, R261H, N300A, and K305A) were analyzed using 30 nM [α-^{32}P] ATP (Perkin Elmer, USA) and 0.2 mM unlabeled ATP. The reactions were run at 37°C or 55°C for 2 hr, stopped by adding equal volumes of 0.5% SDS and 10 mM EDTA, aliquots of the samples spotted onto a polyethyleneimine cellulose TLC (PEI-TLC) plate (Merck, Germany), resolved with 0.4 M LiCl and 1 M formic acid, and the chromatograms analyzed using a BAS 2000 (Fujifilm, Japan) bio-image analyzer quantified using the Image Gauge software program. A fluorimetric real time assay was performed to determine kinetic data for the steady state ATPase activity of TmL (16, 17). To the fluorimeter cuvettes (1 cm path), containing 0.1 M KCl, 50 mM Tris-HCl (pH 8.0), 5 mM $MgCl_2$, 90 μM NADH, and 0.9 mM PEP, was added a mixture containing 5.3 U/mL pyruvate kinase, 7.5 U/mL lactate dehydrogenase, 0.5 mM ATP, and 400 nM TmL at a temperature of 37°C and maintained at 37°C or 55°C for 5 to 20 min at pH 8.0. Excited absorbance at 340 nm was monitored continuously by a spectrophotometer (Shimadzu UV1601, Germany) equipped with a temperature controlled bath (16).

SsDNA binding assay

The ssDNA binding activities of TmL and its mutants were demonstrated by a [γ-^{32}P] ATP labeling method (18) in which polynucleotide kinase (TAKARA, Japan) was used to label the 5'-end of a 30-mer ssDNA (10 nM) with 30 nM [γ-^{32}P] ATP and the labeled ssDNA purified using a Micro-spin G-25 column (GE Healthcare, Denmark) and the phenol extraction method TmL (0-0.2μM) was then incubated with 1 μM of unlabeled and labeled ssDNAs for 1 hr at 37°C or 55°C. The relative ssDNA binding activities for 0.2 μM of TmL and its mutants (E35A, R101F, R261H, N300A, and K305A) in the presence of nucleotides were assessed using an electrophoretic mobility shift assay (EMSA) with the results analyzed by a BAS 2000 Bio-Image analyzer and quantified using the Image Gauge program.

TmS-TmL-mismatched DNA interaction

The formation of the protein-DNA complex was measured by EMSA. For this, G:T mispaired dsDNAs were synthesized by annealing two synthetic 30-mers (5'-AGCTGCCAGGCACC AGTGTCAGCGTCCTAT-3' and 5'-ATAGGACGCTGACATTG GTGCCTGGCAGCT-3'). The reaction mixtures (20 μL) of 25 mM HEPES-NaOH (pH 7.5), 50 mM KCl, 5 mM $MgCl_2$, 0.1 mM DTT, 0.1 mM EDTA, 5% glycerol, 1 mM ADP, 1 mM ADPnP, 2 μM 30-bp mismatched DNA, 2 μM TmL, and 4 μM TmS protein were incubated at 37°C for 1 hr, soaked with SYBR green DNA staining dye (Molecular Probe, USA), washed twice with ddH_2O, and the gel visualized using a UV illuminator (TFX-20.C, France).

Acknowledgements

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