

## Mutational Analysis of Bacterial NAD<sup>+</sup>-dependent DNA Ligase: Role of Motif IV in Ligation Catalysis

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**Abstract** The bacterial DNA ligase as a multiple domain protein is involved in DNA replication, repair and recombination. Its catalysis of ligation can be divided into three steps. To delineate the roles of amino acid residues in motif IV in ligation catalysis, site-directed mutants were constructed in a bacterial NAD<sup>+</sup>-dependent DNA ligase from *Thermus sp.* TAK16D. It was shown that four conserved residues (D286, G287, V289 and K291) in motif IV had significant roles on the overall ligation. Under single turnover conditions, the observed apparent rates of D286E, G287A, V289I and K291R mutants were clearly reduced compared with that of WT ligase on both match and mismatch nicked substrates. The effects of D286E mutation on overall ligation may not only be ascribed to the third step. The G287A mutation has a major effect on the second step. The effects of V289I and K291R mutation on overall ligation are not on the third step, perhaps other aspects, such as conformation change of ligase protein in ligation catalysis, are involved. Moreover, the amino acid substitutions of above four residues were more sensitive on mismatch nicked substrate, indicating an enhanced ligation fidelity.

**Keywords** NAD<sup>+</sup>-dependent DNA ligase; site-directed mutation; single turnover; catalytic kinetics

DNA ligases are involved in DNA replication, repair and recombination from prokaryotic to eukaryotic organisms, and they catalyze the phosphodiester bond formation of single-stranded nicks in double-stranded DNA [1,2]. DNA ligases need a cofactor and a divalent metal ion for ligation catalysis, and can be classed into two types based on their need for either NAD<sup>+</sup> or ATP [3]. ATP-dependent DNA ligases are usually present in eukaryotic organisms such as mammals, yeast, viruses, archaea and bacteriophages [4–6], as well as some eubacteria [7–9]. However, NAD<sup>+</sup>-dependent ligases are primarily found in eubacteria [2,3].

Both the ATP-dependent and NAD<sup>+</sup>-dependent ligases share a common structure that consists of a series of conserved domains including an adenylation domain, OB (oligonucleotide binding) fold, zinc finger, helix-hairpin-helix motif and BRCT domain [3,10]. All DNA ligases catalyze phosphodiester bond formation through a series of similar mechanistic steps. The catalysis mechanism of NAD<sup>+</sup>-dependent ligases involves three independent steps.

First, an adenylation of the Lys present within the essential KXDG motif of the adenylation domain occurs by way of the adenylate portion of an NAD<sup>+</sup> molecule. Second, the AMP moiety from ligase protein is then transferred to the 5'-terminal phosphate present at the single-stranded nick. The third step involves phosphodiester bond formation by a nucleophilic attack of the 3'-hydroxy terminus present at the other side of the gap [1,2].

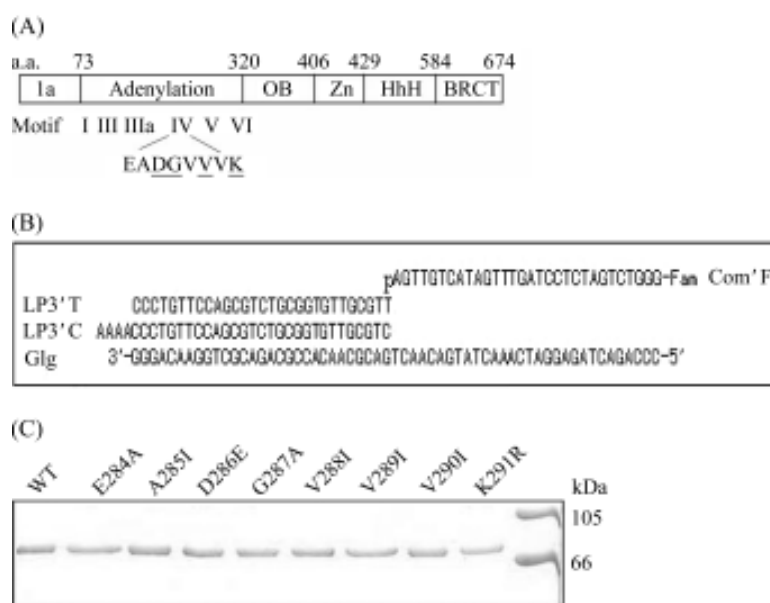
The adenylation domain consists of six conserved motifs (I–VI) [Fig. 1(A)] [3,10]. In these regions, many residues are known to be important for catalysis [11–16]. An ATP- or NAD-binding pocket is organized with the conserved residues dispersed in the adenylation domain [17–19], leading to the formation of a catalytic core that is conserved in a nucleotidyl transferase superfamily, the members of which include DNA and RNA ligases, and RNA capping enzymes [20].

Previous studies have shown that the NAD<sup>+</sup>-dependent DNA ligase from *Thermus sp.* TAK16D is a thermostable protein with high fidelity in DNA ligation [21], and can be used to detect the single nucleotide mutations in cancer

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**Fig. 1 Analysis of site-directed mutants at motif IV of TAK DNA ligase**

(A) Domain organization of TAK DNA ligase. a.a., amino acid; HhH, helix-hairpin-helix motif; OB, oligonucleotide binding fold; Zn, zinc finger. (B) Oligonucleotide substrates used in ligase activity assays. Match nicked substrate (G/C) was annealed with oligo LP3'C, Com3'F and Glg. Mismatch nicked substrate was annealed with oligo LP3'T, Com3'F and Glg, resulting in 3' mismatch (T/G) at the nick site. (C) Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis. Eight mutants and WT ligase were analyzed on a 15% SDS-PAGE gel and visualized with Coomassie Brilliant Blue staining.

related genes [22]. To delineate the roles of residues in motif IV on ligation steps, a set of site-directed mutants of this DNA ligase were created. Ligation activities were assayed under single turnover conditions to determine the effect of single amino acid substitution within motif IV on ligation catalysis.

## Materials and Methods

### Reagents, media and strains

Routine chemical reagents were purchased from Sigma-Aldrich (St. Louis, USA). Restriction enzymes were obtained from New England Biolabs (Beverly, USA). *Pfu* DNA polymerase was from Stratagene (La Jolla, USA). *Escherichia coli* BL21(DE3) cells and plasmid pET11c were obtained from Novagen (Madison, USA). Hitrap columns were purchased from Amersham Bioscience (Piscataway, USA).

### Site-directed mutagenesis at motif IV of the DNA ligase

Site-directed mutagenesis was carried out according to the instruction manual of the Quikchange mutagenesis kit

(Stratagene). The pEV5-Tak plasmid containing the DNA ligase gene was used as the template for initial incorporation of mutant sequences. The mutagenized plasmids were transformed into *E. coli* JM109 competent cells. The entire DNA ligase gene region was sequenced to confirm that only a desired site-directed mutation was introduced. A mutation-containing fragment was generated by *Nco*I and *Bam*HI digestion and subcloned into pET11c. The resulting plasmids were transformed into *E. coli* strain BL21(DE3).

### Expression and purification of mutation proteins

The expression and purification of the mutant proteins were carried out as previously described [23]. Briefly, an overnight culture of *E. coli* strain containing each of the desired mutations was diluted 100-fold into 1 liter of LB medium. The *E. coli* strain was grown at 37 °C while shaken at 200 rpm until the optical density at 550 nm reached approximately 0.6. After adding IPTG to a final concentration of 1 mM, the culture was incubated with shaking for extra 3.5–4 h to induce protein expression. The induced *E. coli* cells were harvested by centrifugation at 2057 g for 10 min, washed with phosphate-buffered saline (PBS) and stored at –20 °C prior to use.

Cells were sonicated in PBS and centrifuged, the super-

nantant was heated at 70 °C for 30 min to denature *E. coli* proteins. The proteins in the heat-treated supernatant were precipitated by adding ammonium sulfate to 80% saturation. The precipitate was dissolved in 10 ml of butyl buffer A [20 mM Tris-HCl (pH 8.0), 1 mM EDTA, 0.2 mM dithiothreitol (DTT), 3 M NaCl]. The sample was loaded onto a 5 ml HiTrap Butyl FF column, and the proteins were eluted with 6 column volumes (CV) of a descending linear gradient of NaCl (3–0 M) in the same buffer on an Amersham AKAT Purifier chromatography system. Fractions containing ligase protein were pooled and dialyzed against Hitrap Blue buffer A [20 mM Tris-HCl (pH 8.0), 0.5 mM EDTA, 0.5 mM DTT]. The sample was loaded onto a 1 ml Hitrap Blue column. The protein was eluted with 10 CV of an ascending linear gradient of NaCl (0–1 M). Fractions containing ligase protein (0.35–0.50 M NaCl) were pooled and kept at –20 °C in stock buffer [50 % glycerol, 20 mM Tris-HCl (pH 8.0), 1 mM EDTA (pH 8.0), 1 mM DTT, 0.1 mg/ml bovine serum albumin (BSA)]. Protein concentrations were determined by quantifying the intensity of protein bands on 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel using BSA as a standard.

### Substrates and ligation assays

The oligonucleotide match nicked substrate was formed by annealing two oligonucleotides (33 mer for LP3'C and 30 mer for Com3'F) with a 59 mer complementary oligonucleotide (Glg) [Fig. 1(B)]. Oligonucleotides LP3'C and Glg were in 1.2-fold excess to Com3'F so that all the 3' Fam-labeled Com3'F represented nicked substrates. A substrate with a mismatch at the 3' position of a nick was formed by annealing LP3'T and Com3'F to the complementary strand (Glg) [Fig. 1(B)]. The nicked DNA duplex substrates were formed by denaturing DNA oligonucleotides at 94 °C for 2 min followed by re-annealing at room temperature for at least 2 h in TE buffer (Tris-HCl, pH 8.0, 1 mM EDTA).

For preparing the adenylation substrate, a gapped and nicked DNA duplex (made from LP3'C-1, Com 3' F and Glg as above) was incubated with T4 DNA ligase in the presence of ATP and Mg<sup>2+</sup> at 37 °C for 1 h. The resulting duplex oligonucleotide was separated on 15% sequencing PAGE gel. The adenylation oligonucleotide (Com3'F) shown under ultraviolet light was cut out of the gel and purified by soaking the gel in elution buffer [0.5 M (NH<sub>4</sub>)<sub>2</sub>Ac, 10 mM MgAc, 1 mM EDTA, 0.1% SDS] and gentle shaking overnight at room temperature. The concentration of adenylated Com3'F was determined on 10% sequencing PAGE gel using the Com3'F as standard.

Ligation mixtures containing proper amounts of DNA ligase, 10 nM 3'-Fam-labeled substrate, 20 mM Tris-HCl (pH 7.6), 100 mM KCl, 10 mM DTT, 20 µg/ml BSA, 10 mM MgCl<sub>2</sub> and 1 mM NAD<sup>+</sup> were incubated at 65 °C for various times. At each time point, 10 µl of reaction mixture was withdrawn and immediately mixed with an equal volume of loading buffer (1% blue dextran, 80% formamide, 50 mM EDTA) to terminate the reaction. Samples (3.5 µl) were denatured for 3 min at 95 °C prior to separation on the 10% sequencing gel according to the instruction manual on an ABI PRISM 377 sequencer (Applied Biosystems, Foster City, USA). Results were quantified using GeneScan software Version 3.0 (Applied Biosystems).

## Results

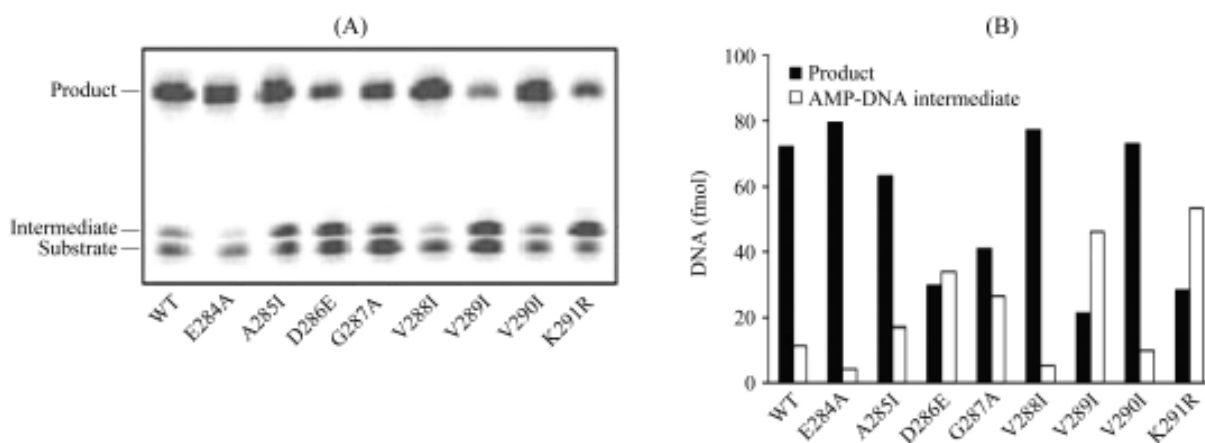
### Construction and protein expression of site-directed mutants at motif IV

NAD<sup>+</sup>-dependent DNA ligase is a conserved family with the same domain organization [3,10]. Motif IV within the adenylation domain of the DNA ligase from *Thermus sp.* TAK16D consists of EADGVVVK [Fig. 1(A)]. So we intend to identify the amino acid residues at motif IV that might be involved in DNA ligation catalysis. Four highly conserved amino acid residues, as well as their flanked residues, in this NAD<sup>+</sup>-dependent ligase were chosen for site-directed mutagenesis.

Eight site-directed mutants were made by polymerase chain reaction using the QuikChange mutagenesis kit. The desired mutation genes confirmed by sequencing were overexpressed in *E. coli*. The purified ligase proteins migrated normally on SDS-PAGE gel [Fig. 1(C)]. The ligation activity was analyzed using match or 3' mismatch nicked DNA substrate that was annealed by three oligonucleotides, one labeled with fluor Fam at its 3' end [Fig. 1(B)]. The fluorescent ligation product (63 mer) can be separated from the unligated substrate (30 mer) on a sequencing gel.

### Effect of site-directed mutants in motif IV on match nicked substrate

The ligation activities of the eight mutants along with the WT ligase were initially assayed using the enzyme: substrate ratio of 1:1 (E:S=1:1, S=10 nM G/C match nicked substrate). As shown in Fig. 2(A), the WT ligase, as well as E284A, V288I and V290I, showed almost normal ligation activity on the nicked substrate with less AMP-



**Fig. 2 Ligase activity analysis of eight site-directed mutants at motif IV of TAK DNA ligase**

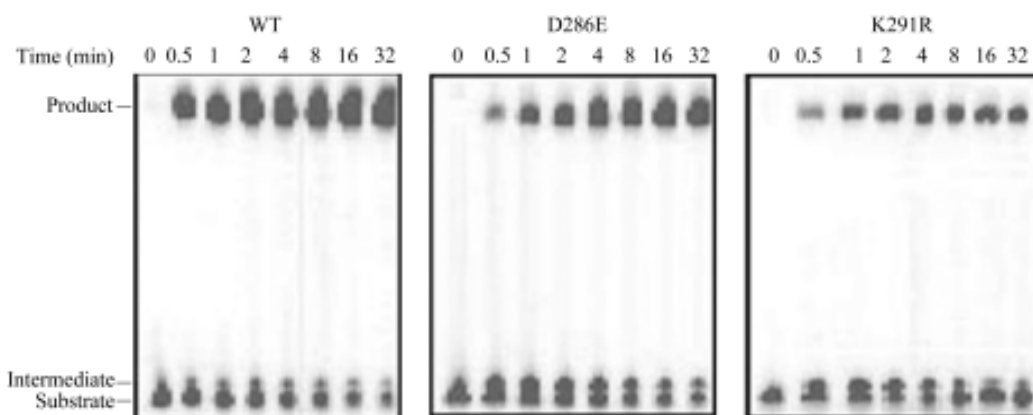
(A) Sequencing gel picture of a representative ligation activity assay. Ligation assays were carried out as described in “Materials and Methods” with 10 nM ligase protein and 10 nM match nicked substrate at 65 °C for 15 min. (B) Quantitative analysis of ligation activity with 10 nM ligase protein and 10 nM match nicked substrate at 65 °C for 15 min.

DNA intermediate after 15 min incubation at 65 °C. Under the same conditions, D286E, G287A, V289I and K291R mutants showed reduced ligation product, less than 40% of that of the WT ligase, and accumulated AMP-DNA intermediate to relevant extent [Fig. 2(B)].

Further kinetic analysis using match nicked substrate was carried out under single turnover conditions (E:S=5:1) to discern the potentially similar or different behavior in ligation reactions of the mutant DNA ligase. Fig. 3 shows the representative gel pictures of kinetic analysis for WT ligase, D286E and K291R. The data were extracted and used to fit the double exponential growth model. As shown in Fig. 4, the overall kinetic curves could be divided into a

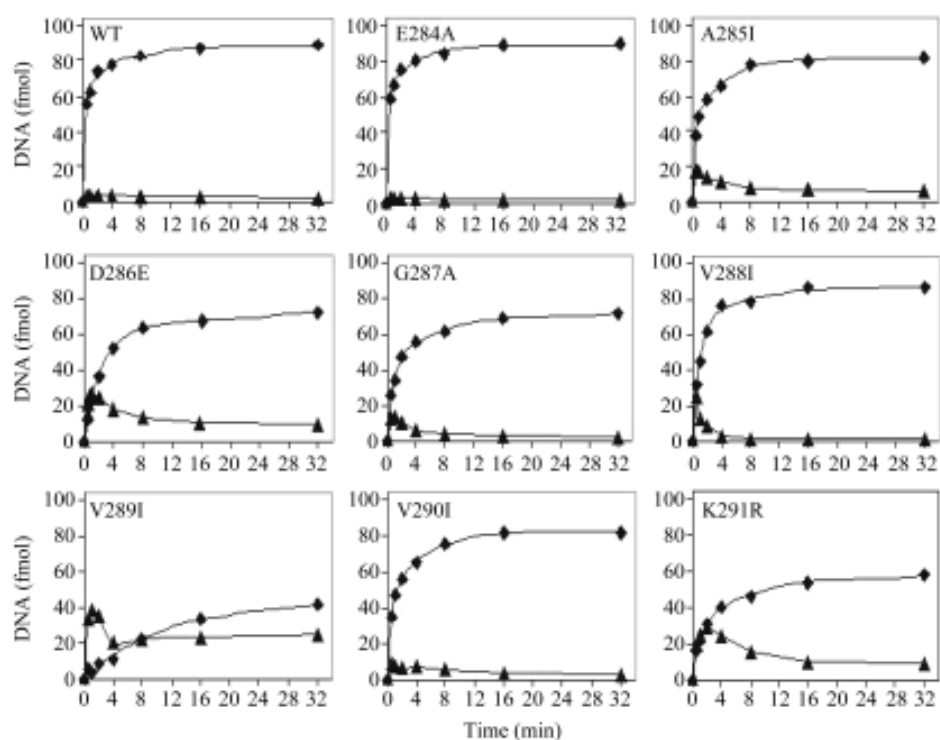
short, quickly increasing phase and a very slow growing phase. So the apparent rate constant of the  $K_{obs}$  value in corresponding to the first phase could be used to illustrate the ligation catalysis rate. Kinetic formation of AMP-DNA intermediate was also shown in Fig. 4 for each mutant and WT ligase.

The  $K_{obs}$  values for each mutant and WT ligase are summarized in Table 1. WT ligase completed approximately 90% product in 32 min under this condition with  $K_{obs}$  = 3.83 min<sup>-1</sup>. E284A showed WT-type behavior in kinetics with a  $K_{obs}$  value of 3.59 min<sup>-1</sup>, and the product formation grew quickly and reached the plateau within the first 4 min. The four mutants of conserved residues (D286E,



**Fig. 3 Sequencing gel pictures of representative kinetic analysis of ligation activity of WT ligase and two site-directed mutants at motif IV of TAK DNA ligase**

Ligation assays were carried out as described in “Materials and Methods” with 50 nM ligase protein and 10 nM match nicked substrate at 65 °C for indicated times.



**Fig. 4** Kinetic analysis of TAK DNA ligase and site-directed mutants at motif IV under single turnover conditions using match nicked substrate

The reaction mixtures containing 50 nM ligase protein, 10 nM match nicked substrate, and other components as described in “Materials and Methods” were incubated at 65 °C. The diamonds (◆) represent product formed at the indicated time point, which were used to fit the double exponential growth model; triangles (▲) represent AMP-DNA intermediate.

**Table 1** Apparent rate constants of mutants and WT ligase

Group	$K_{\text{obs}}$ ( $\text{min}^{-1}$ )	
	G/C	T/G
WT	3.83	1.18
E284A	3.59	0.87
A285I	2.85	0.26
D286E	0.82	ND
G287A	1.33	ND
V288I	2.46	0.82
V289I	0.09	ND
V290I	2.61	0.71
K291R	0.96	0.0017

Ligase activity assays were carried out under single turnover conditions with 50 nM ligase protein and 10 nM match (G/C) or mismatch (T/G) nicked substrate at 65 °C. ND, the ligation product formed was too low to be measured.

G287A, V289I and K291R) formed less ligation product during the time-course, completing 72.4%, 72.1%, 41.7% and 58.0% product, respectively, with  $K_{\text{obs}}$  values of

0.82, 1.33, 0.09 and 0.96  $\text{min}^{-1}$ , respectively (Table 1). The product formation of A285I, V288I and V290I increased at a moderate rate (Table 1), reaching a plateau within approximately 8 min. After 32 min incubation, A285I, V288I and V290I completed 81.8%, 86.8% and 82.4% product, respectively.

WT ligase and eight mutants accumulated AMP-DNA intermediate on the match nicked substrate through the time-course (Fig. 4). The accumulated intermediate reached a peak at approximately 2 min then maintained a plateau, indicating that the first step of adenylation was fast. In addition, the self-adenylation of the eight mutants and WT ligase showed no obvious difference (data not shown). For the eight mutants and WT ligase, the amounts of accumulated AMP-DNA intermediate were different. G287A, as well as E284A, V290I and WT ligase accumulated less intermediate, indicating that G287A might affect the secondary step (transferring AMP moiety from protein to DNA). D286E, V289I and K291R accumulated a much higher amount of intermediate, and their overall ligation rate was reduced, simply illustrating that these three mutants affected the third step (nick-joining).

### Effect of eight mutants on nick-joining at pre-adenylated match substrate

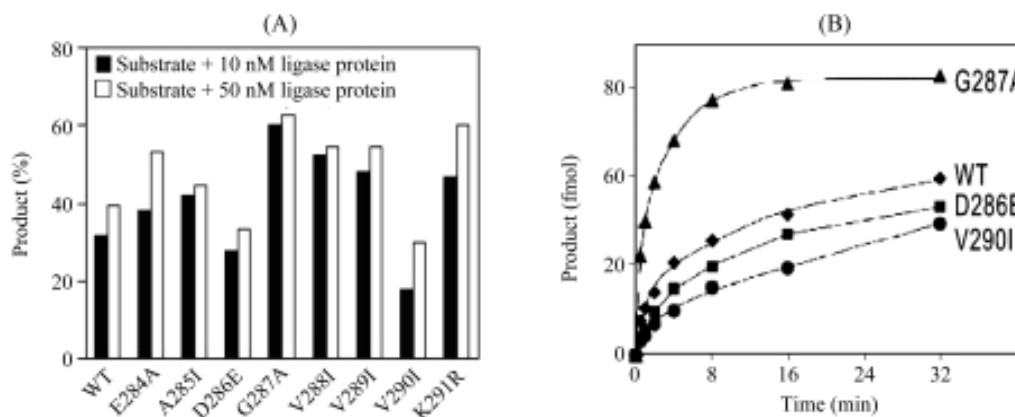
Previous investigations have shown that G161 (corresponding to D286 in this DNA ligase) at motif IV in *Chlorella* virus DNA ligase plays a dominant essential role in the first step (self-adenylation) and only a supportive “important” role in the third step [15]. This led us to carry out a series of ligation assays using pre-adenylation substrate to examine the effects of the eight mutants on the third step.

NAD<sup>+</sup>, even at low concentration, completely inhibited nick-joining by NAD<sup>+</sup>-dependant DNA ligase (data not shown). In the assays, E:S=1:1 and E:S=5:1 were used to examine the effect of eight mutants on the third step. WT ligase and its eight mutants were not able to completely seal the nicked pre-adenylated substrate after 30 min incubation at 65 °C. As shown in **Fig. 5(A)**, WT ligase formed only 32% and 39% product under E:S=1:1 and E:S=5:1, respectively. The nick-joining ligation of the pre-adenylation match substrate catalyzed by D286E was a slightly less than the WT ligase, indicating that this residue might affect the third step, although only to a limited extent. However, V290I yielded less product in comparison with WT ligase, with 17.8% and 29.8% product under E:S=1:1 and E:S=5:1, respectively. Unexpectedly, the other mutants formed ligation products more than WT did [**Fig. 5(A)**]. In particular, mutant G287A greatly enhanced nick-joining (the third step) product in comparison with WT ligase. To confirm the above results, WT ligase and three mutants were selected for kinetic analysis under E:S=5:1 (S=10 nM). As shown

in **Fig. 5(B)**, G287A catalyzed nick-joining of the pre-adenylation substrate much faster than WT ligase, and the apparent rate constant ( $K_{obs}=2.75 \text{ min}^{-1}$ ) was more than twice that of WT ligase ( $K_{obs}=1.06 \text{ min}^{-1}$ ). D286E and V290I showed WT-lower type of catalytic kinetics with  $K_{obs}$  of  $0.58 \text{ min}^{-1}$  and  $0.43 \text{ min}^{-1}$ , respectively, confirming that these two mutants might affect the third step. However, the other mutants in motif IV do not play an essential role in the third step, although some mutants, such as G287A, V289I and K291R, reduced overall ligation activities. Therefore, their effect on overall ligation catalysis might involve other aspects of the overall ligation.

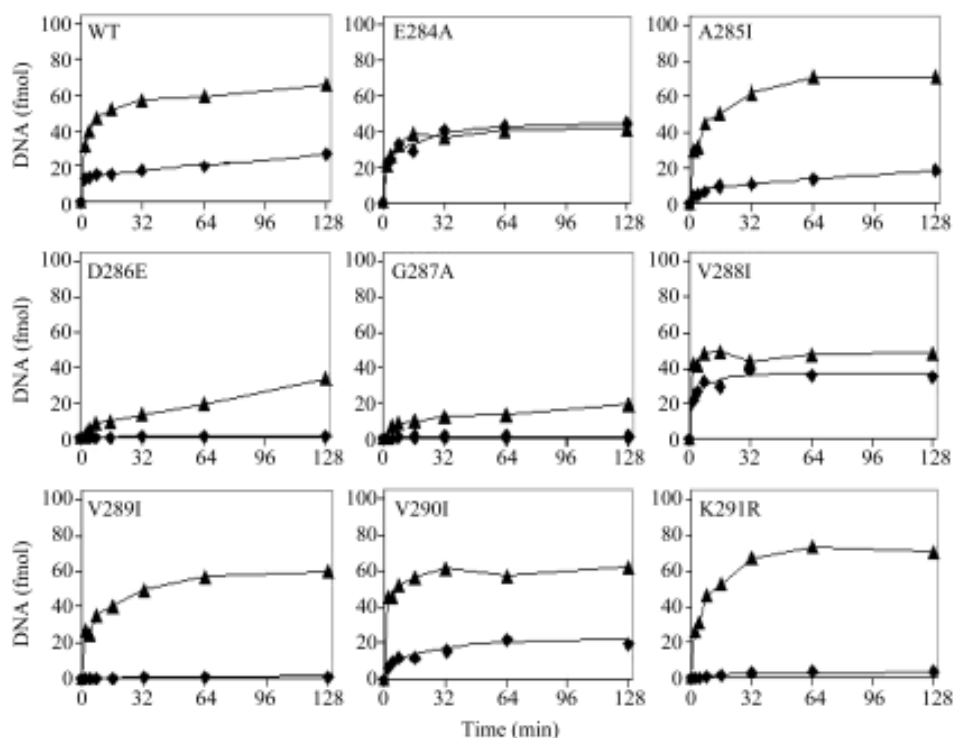
### Effects of site-directed mutation on ligation fidelity

All the mutants maintained at least a 40% level of WT ligation activity on the match nicked substrate [**Fig. 2(B)**]. Given the significant effects observed on some site-directed mutations, their ligation activities on mismatch nicked substrates might change due to amino acid substitutions. To investigate this possibility, kinetic analysis using 3' mismatch nicked substrates [**Fig. 1(B)**] was carried out under single turnover conditions (E:S=5:1). The data were used to fit the double exponential growth model. After 128 min incubation, WT ligase finally yielded 27.1% 3' mismatch ligation product with  $K_{obs}=1.18 \text{ min}^{-1}$ , and accumulated AMP-DNA intermediate to a huge extent. Under the same reaction conditions, E284A, A285I, V288I and V290I generated a similar amount of 3' mismatch ligation product as the WT ligase at the final time point (**Fig. 6**), and catalyzed the ligation reaction of mismatch nicked substrate at a lower rate in comparison to WT ligase (**Table 1**). However, AMP-DNA intermediate accumulated and steadily



**Fig. 5** Ligation activity analysis of WT ligase and site-directed mutants on pre-adenylated substrate

(A) Quantitative analysis of ligation activity with 10 nM or 50 nM ligase protein and 10 nM pre-adenylated substrate at 65 °C for 30 min. (B) The kinetic analysis of WT ligase and three mutants on pre-adenylated substrate using 50 nM ligase protein, 10 nM pre-adenylated substrate, and other components as described in “Materials and Methods” were incubated at 65 °C for indicated time.



**Fig. 6** Kinetic analysis of TAK DNA ligase and site-directed mutants at motif IV under single turnover conditions using mismatch nicked substrate

The reaction mixtures containing 50 nM ligase protein, 10 nM mismatch nicked substrate, and other components as described in “Materials and Methods” were incubated at 65 °C. The diamonds (◆) are representative of product formed at indicated time points, which were used to fit the double exponential growth model; triangles (▲) are representative of AMP-DNA intermediate.

increased during the time-course, indicating a defect in the third step for the mismatch nicked substrate.

D286E and G287A formed significantly less mismatch ligation product (less than 2% at the final time point), and accumulated less AMP-DNA intermediate than WT ligase (Fig. 6). The results illustrated that D286E and G287A might affect the adenylation or AMP transfer to DNA substrate, as well as the third step on mismatch nicked substrate. V289I and K291R yielded much less mismatch ligation product too, but accumulated relevant intermediate comparable to WT ligase (Fig. 6), indicating that V289I and K291R generated an impact on the third step on the mismatch nicked substrate.

Previously, the ligation fidelity was judged using the ratio of two kinds of initial velocity of ligation catalysis on match and mismatch nicked substrate [24]. In this work, the ratio of two  $k_{\text{obs}}$  values of ligation kinetics on match and mismatch nicked substrate were used to show the ligation fidelity. The ratio of  $k_{\text{obs}}$  values for match and mismatch nicked substrate was more than 500, indicating that mutant K291R enhances the ligation fidelity, in agreement with

the previous report [24]. Because much less product was formed,  $k_{\text{obs}}$  values for D286E, V289I and G287A could not be measured. It is obvious that the ligation fidelity for these three mutants was enhanced much more than for K291R. These data show that substitution of conserved residues at motif IV could increase the ligation fidelity.

## Discussion

Both ATP- and NAD<sup>+</sup>-dependent DNA ligases are multi-domain proteins. The adenylation domain is thought to comprise a catalytic active center, including self-adenylation, transferring of AMP moiety, and nick-joining [2,10]. The adenylation domain is further divided into six conserved motifs [Fig. 1(A)] in NAD<sup>+</sup>-dependent DNA ligase. A conserved K in motif I (KXDG) is shown as an AMP recipient in ATP- and NAD<sup>+</sup>-dependent DNA ligases [11,25]. Several residues dispensed within the adenylation domain in some DNA ligases have been mutated to delineate their roles in the catalytic mechanism [11–16]. The

conserved amino acid residues of motif IV consist of DGxVxK in NAD<sup>+</sup>-dependent DNA ligases [10]. K294R in Tth ligase has been previously shown to enhance the ligation fidelity [24].

The NAD<sup>+</sup>-dependent DNA ligase from *Thermus sp.* TAK16D is a thermostable protein, which was reported as a high fidelity DNA ligase, and can be used to detect single nucleotide mutations in cancer-related genes [21]. To investigate the role of motif IV in ligation function, we created eight site-directed mutants in motif IV of the DNA ligase. This work shows that four conserved residues (D286, G287, V289 and K291) play an important role in DNA ligation. Under single turnover conditions (E:S=5:1), the ligation activity of D286E, G287A, V289I and K291R was reduced with a  $K_{obs}$  value lower than that of WT ligase (**Table 1**). In Tth DNA ligase, K294R mutant (equal to K291R in this ligase) was reported to maintain WT activity [11], perhaps due to use of excess ligase.

In a virus DNA ligase, E161 (corresponding residue of D286 in this ligase) also affects the third step. But we thought the major role of E161 is included in the first step of adenylation because removal of the charged group by substitution of Ala totally abolished adenylation, as well as the whole ligation activity [15]. Even a conserved substitution of E161D could not rescue the ligation activity to a relevant level. So the acidic group in the residue is essential for ligation activity. Self-adenylation of ligase protein is metal-dependent [26]. And with T4 DNA ligase, kinetic analysis has shown that the adenylation is according to a two-metal ion mechanism [27]. The crystal structure of Tfi DNA ligase shows that D283 (correspondive to D286 in this ligase) is around the AMP-binding pocket and might participate in coordination of the divalent metal ion [17]. In fact, the nick-joining activity of D286E on pre-adenylated substrate was not greatly reduced in comparison with WT ligase (**Fig. 5**). Using pre-adenylated DNA substrate, the nick-joining activity of human DNA ligase I was inhibited by ATP, indicating that the AMP-binding pocket in ligase is available to complete the reaction [26]. A similar observation was illustrated in the NAD<sup>+</sup>-dependent DNA ligase in this work (data not shown). Based on the structure and mutagenesis analysis, D286 is likely involved in coordination with metal in the overall ligation catalysis and particularly in the first step. Available evidence has illustrated that the conformation of DNA ligases, particularly in the AMP-binding pocket, is altered after the adenylation ligase is formed, leading to a more compact, active status [28]. Thus, D286 might not be included in metal coordination in the third step due to possible formation of a new metal-binding site. G287A reduced the overall ligation rate (**Fig.**

**4**), but it enhanced the third step by using pre-adenylation substrate [**Fig. 5(B)**]. And this mutant was more sensitive to mismatch nick substrate with greatly reduced ligation product and less accumulation of AMP-DNA intermediate (**Fig. 6**). The effect of the residue G287 on ligation catalysis might be involved in other aspects, perhaps in the second step.

The residues of V286 and K288 in Tfi ligase correspond to V289 and K291 in this ligase, respectively. Both are lined with the AMP moiety in the 3-D structure of Tfi ligase. K288 in Tfi ligase might bridge an ion pair with Glu114 in motif I [17]. In Tth ligase, substitutions of K294 (correspondive to K291) with Arg, Glu and Leu did not abolish the adenylation and transfer of AMP moiety [11]. Our data also showed that binding to the nicked substrate and self-adenylation of those mutants occurred with no obvious difference (data not shown). V289I and K291R are more sensitive to a mismatch at the 3' side of the nick (**Fig. 6**), and did not reduce the third step based on nick-joining on pre-adenylation substrate (**Fig. 5**). As discussed above, the residues of V289 and K291 might be involved in maintaining the conformation of adenylation ligase. Ile substitution of V289 and Arg substitution of K291 does not alter the charge of the side group of residues, except the distance to other residues in the AMP-binding pocket. Those conserved substitution mutations yield an impact on overall ligation catalysis, perhaps through a change in the stability or conformation of adenylation ligase.

Using mismatch nicked substrate, we examined the ligation activity of eight mutants and WT ligase. The four mutants at conserved residues of D286, G287, V289 and K291 showed greatly reduced ligation activity, almost with no mismatch ligation product under this condition during incubation for 128 min. Our unpublished data showed that the binding affinity of those four mutants on the mismatch nicked substrate occurred with no obvious difference. The ability of a ligase to discriminate a mismatch nicked substrate is regulated in both the second and third steps [21,29]. But D286E and G287A accumulate less AMP-DNA intermediate, which indicates that self-adenylation of ligase or transfer of AMP moiety to DNA substrate was defective. The results further confirm that the role of D286 in ligation catalysis is not confined at the third step. In contrast, V289I and K291R accumulated AMP-DNA intermediate in the same way as WT ligase. V289I and K291R enhance their ligation fidelity by lowering nick-joining activity. As discussed above, V289 and K291 might be involved in maintaining the conformation of adenylation ligase. V289I and K291R are more sensitive to 3' mismatch substrate, leading to a great reduction of nick-joining.

In addition, all the mutants of D286E, G287A, V289I and K291R at motif IV enhance the ligation fidelity. With K291R, the same result was achieved in another NAD<sup>+</sup>-dependent DNA ligase as previous studies [24]. This work expands the engineering of a higher fidelity ligase, which is useful in ligase-mediated detection of gene mutation.

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