The Deletion of Amino-Terminal Domain in *Thermoactinomyces vulgaris* R-47 α-Amylases: Effects of domain N on Activity, Specificity, Stability and Dimerization

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*Thermoactinomyces vulgaris* R-47 α-amylases, TVA I and TVA II, have a domain N, which is an extra structure in the family 13 enzymes. To investigate the roles of domain N in TVAs, we constructed TVAs-ΔN mutants which are deleted in domain N, and Y14,16,68A and Y14,16,68A mutants of TVA II. TVA-ΔN were unstable under alkaline conditions, and their thermal stabilities were 10°C lower than those of wild-types. The specific activities of TVAs-ΔN for pullulan, starch, cyclodextrins, and oligosaccharides were drastically decreased, being about 1,500- to 10,000-fold smaller than those of wild-types. The kcat values of Y14,16,68A and Y14,82,95A for all tested substrates were markedly decreased, and the Km value of Y14,16,68A for α-CD and maltotriose were 25- and 3-fold larger, and that of Y14,82,92A for starch was 10-fold larger than that of the wild-type. TVA I and TVAs-ΔN in solution are a monomer, while TVA II is a homo-dimer, calculated by their molecular masses. These results suggest domain N in TVAs is an important structure for stabilization of enzymes, recognition and hydrolysis of substrates, and dimerization of TVA II.

Key words: α-Amylase; TVA; domain N; family 13

α-Amylases (1,4-α-D-glucan-4-glucanohydrolase; EC 3.2.1.1) hydrolyze internal α-(1→4)-glucosidic linkages of starch and glycogen to release α-anomer products. They are widely distributed in nature, and significantly important for starch and glycogen metabolism in microorganisms, plants, and animals. Several α-amylases have been successfully used not only in the fermentation, food, and starch processing industries, but also in the pharmaceutical field as a digestive.1,2

*Thermoactinomyces vulgaris* R-47 produces two α-

amylasses, TVA I (637 amino acids in the mature form, Mr = 74.0 kDa3) and TVA II (585 amino acids, Mr = 67.5 kDa4). In addition to hydrolyzing α-(1→4)-glucosidic linkages of starch to produce mainly maltose, TVA I and TVA II can efficiently hydrolyze α-(1→4)-glucosidic linkages of cyclodextrins (CDs) and pullulan to produce maltose and panose, respectively,5 something most α-amylases cannot do.6 The primary structures of both enzymes also resemble each other. Recently, the crystal structure of TVA II has been determined.7 TVA II is composed of four domains, A, B, C, and N (Fig. 1(A)). Domain A has a (β/α)6 barrel structure, and domain

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Abbreviations: TVA, *Thermoactinomyces vulgaris* R-47 α-amylase; CGTase, cyclodextrin glucanotransferase

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Fig. 1. Crystal Structure of TVA II.

(A) The monomeric structure of the TVA II molecule. Domain N, A/B, and C are shown in gray. The three catalytic residues (Asp325, Glu354, Asp421) are shown in black sticks. An arrow shows the insertion point (between Val121 and Phe122) of a thrombin protease recognition-sequence. (B) Close-up view of domain N in TVA II. The seven β-strands are shown in gray, and Tyr residues are shown in black sticks. Residues shown in thick sticks were replaced with Ala residues in this report. These figures were illustrated by the program MOLSCRIPT.10
B is a small component which protrudes at the third β-strand of domain A. These components are collectively called domain A/B in this paper. Domain N and domain C are located prior to the N-terminus and subsequent to the C-terminus of domain A/B, respectively.

Three-dimensional structures of the family 13 enzymes from animals, plants, and microorganisms have been analyzed and deposited in the Brookhaven Protein Data Bank, for example α-amylases from *Aspergillus oryzae*, *Sulfolobus solfataricus* KM1, and pig pancreas, isomylase from *Pseudomonas amyloferans*, cyclodextrin glucanotransferases (CGTase) from *Bacillus steaethophilus*, *Bacillus circulans* strain 8, and *Bacillus circulans* strain 251, and maltegenic amylase from *Thermus* strain M6501. The fundamental structure of the family 13 enzymes is composed of three common domains, domain A/B and C. CGTase has two extra domains called domain D and domain E subsequent to the C-terminus of domain C, while *Pseudomonas* isomylase, *Thermus* maltegenic amylase and *Sulfolobus* α-amylase are reported to have a domain prior to the N-terminus of domain A/B like TVA II. The roles of domain A/B and domain C have been elucidated. Domain A/B has the active center including the catalytic residues and substrate-binding residues, and is the most important domain for the enzymatic activity. Domain C seems less important for the activity, in fact, Odhan et al. reported that domain C had no effect on the enzymatic activity. Little is known, however, about domain N, and its roles in the enzymatic activity.

In this report, we tried to genetically truncate the N-terminus of TVA I and TVA II. To obtain the domain N deleted mutants, TVA I-ΔN and TVA II-ΔN, we constructed TVA I-Tn and TVA II-Tn mutants in which was inserted a thrombin protease recognition site between domain N and domain A/B to separate the domains after protein expression. Many aromatic residues are located in domain N of TVA II, therefore we constructed Y14,16,68A and Y41,82,95A-TVA II mutants to show the effects of the aromatic residues on substrate-recognition (Fig. 1(B)). We examined their enzymatic properties and kinetic parameters to investigate the roles of domain N in TVA I and TVA II.

**Materials and Methods**

**Substrates of the enzymes.** Soluble starch was purchased from Merck, Germany. Pullulan (Mw = 72,500), amyllose (DP = 17), and a series of maltotriosylsaccharides from maltotriose to maltotrihexaose were obtained from Hayashibara Biochemical Laboratories, Japan. We used maltotriosylsaccharides reduced by NaBH₄, α-, β-, and γ-Cyclodextrins were obtained from Nihon Shokuhin Kako, Japan.

**Gene construction of TVA I and TVA II mutants.** The enzymes were prepared from recombinant *Escherichia coli* MV1184 cells. Oligonucleotide-directed mutagenesis was done using a Mutan-K *in vitro* mutagenesis kit (Takara, Japan) according to the method of Kunkel for the construction of an expression plasmid of TVA I, pTV93 and TVA II, pTN-302-10. TVA I-Tn and TVA II-Tn mutants were constructed to insert the primers, 5' - CGG CGT CGG CTT TTT GAA GGA TCC ACG CGG AAC CAG ATT GGG AAT AAT ATA AAA-3' and 5' - AGA ACC CCG CGG AAC CAG CAC TTC GCT CGG GTG GAT GT-3', coded amino acid sequences recognized by thrombin protease, between domain N and domain A/B (between Asn123 and Phe124 of TVA I, between Val121 and Phe122 of TVA II). Y14,16,68A mutant was constructed using two primers; 5' - CTT TTC CGA GAT CGG GCC AGC AGC GCT TGC TTT CGG TTC TTC-3' and 5' - TTC CAG TAA AGC TTC AAA AGC ATC AAA TCG CTC-3', and Y41,82,95A mutant using three primers; 5' - AGC ATA GCG GTC TGC AGC GAG CAC CTC GCA-3', 5' - CAA CAA AAA CAC GGC TTT CAC CCG TTT GGT GGA AC-3' and 5' - ACC GGT CTC GGC GAA TGC AAC AGC CTC CCC TTG-3'. These mutants were expressed in *Escherichia coli* MV1184 cells.

**Preparation of wild and mutated enzymes.** Wild-type TVA I and TVA II were purified as described. TVA I-ΔN and TVA II-ΔN mutants were purified with a different buffer solution because the enzymes were unstable under alkaline conditions. The elution buffer was changed from Tris-HCl buffer (pH 7.5) to sodium phosphate buffer (pH 6.0). Y14,16,68A and Y41,82,95A mutants were not elutable from a hydrophobic column, therefore we used an anion-exchange column. SDS-PAGE gave a single band for each of the purified enzymes. The protein concentrations were measured by the method of Lowry et al. using bovine serum albumin (Wako Pure Chemicals, Japan) as a standard.

**Enzymatic assays of TVA I-ΔN and TVA II-ΔN mutants.** The activity was assayed as described. The purified TVA I-ΔN or TVA II-ΔN mutant (concentrated to 1.5 mg/ml, 50 μl) was added to 0.5% (w/v) of each substrate (200 μl) in 100 mM sodium phosphate buffer (at optimum pH), and the hydrolysis reaction started at optimum temperature for 300 min. After the reaction, the method of Somogyi and Nelson was followed. One unit of substrate-hydrolyzing activity was defined as the amount of enzyme that catalyzed the hydrolysis of 1 μmol of glucosidic linkages per minute. For maltotriose, the glucose produced was measured by the method using a glucose oxidase-peroxidase, GOD-POD. (24)
Kinetic studies of Y14,16,68A and Y41,82,95A mutants. The kinetic parameters of Y14,16,68A and Y41,82,95A mutants for various substrates were measured as follows. Purified Y14,16,68A or Y41,82,95A mutant (diluted to 0.01 mg/ml, 120 μl) was added to various concentrated substrates (480 μl) in 100 mM sodium phosphate buffer (pH 6.0), and the hydrolysis reaction started at 40°C, with sampling every 5 minutes. After the reaction had stopped, the method of Somogyi and Nelson\(^{[23]}\) or the method of GOD-POD\(^{[24]}\) was followed.

Analysis of hydrolysis products. The hydrolysis patterns of wild and mutated TVAs on various substrates were identified as follows. Purified enzyme (50 μl) was added to 1% \(w/v\) of each substrate (200 μl) in 100 mM sodium phosphate buffer (at optimum pH), and the hydrolysis reaction started at 40°C, with sampling at 1, 3, 6, and 24 hours later. The reaction products were separated by thin layer chromatography (TLC) on silica gel 60 (Merck) with \(n\)-butanol/ethanol/water \(5/5/3\), and the spots were detected by charring with \(H_2SO_4\).\(^{[25]}\)

Molecular mass measurements of TVAs and TVAs-ΔN. The molecular masses of purified TVAs and TVAs-ΔN were calculated from the amino-acid sequences, SDS-PAGE, and gel filtration. Gel filtration was done by size exclusion column chromatography (Superose HR12 10/30 column was used) based on the FPLC system (Pharmacia). The proteins (concentrated to 1.0 mg/ml, 100 μl injection) were eluted with 50 mM phosphate buffer (pH 6.0) containing 100 mM NaCl, and the flow rate was 0.5 ml/min. The size marker proteins from 290 kDa to 12.4 kDa (Oriental Yeast Inc.) and purified \(A. oryzae\) α-amylase (TAA) as a standard protein were used.

Results

Expression of TVA I-ΔN and TVA II-ΔN mutants

To genetically truncate the N-terminus of mature TVA I, plasmids encoding TVA I including deletion mutants of the N-terminal 7, 10, 11, 12, 16, and 19 amino acid residues were constructed. The mutants with up to 12 amino acids deleted were expressed and soluble, their hydrolysis patterns of starch and pullulan were identical to those of wild-type enzyme (data not shown), while the mutants with 16 and 19 amino acid residues deleted were not expressed. These results suggest that the amino acid residues between 12 and 16 from the N-terminus have an essential role for the protein expression or folding, and the soluble truncated enzymes could not be obtained by this method.

Domain N was, therefore, proteolytically removed. A sequence composed of six amino acids, Leu-Val-Pro-Arg-Gly-Ser, the cleaving site of thrombin protease, was genetically inserted between domain N and domain A/B of TVA I and TVA II, and the enzymes harboring the resulting plasmids were cleaved. The results of SDS-PAGE showed that the enzymes were, however, digested in \(E. coli\) during cultivation. These proteolytic enzymes were purified and electrophoresed on a 10% SDS/polyacrylamide gel. Their molecular masses were about 10–15 kDa smaller than those of wild-type TVA I and TVA II (Fig. 2(A), (B)), which agree with their calculated molecular masses without domain N. The proteolytic fragments of 10–15 kDa were not observed in the crude enzyme on SDS-PAGE, and could not be purified.

To confirm that domain N was lost in the proteolytic enzymes, the N-terminus was sequenced. In both enzymes, the sequence was Arg-Gly-Ser-Phe-Thr-, indicating the proteolytic digestion of the thrombin protease recognizing sequence, although the cleaving sites were one amino acid different from that of thrombin protease (thrombin protease cleaves between Arg and Gly\(^{[26]}\)). We designated these enzymes as TVA I-ΔN and TVA II-ΔN, respectively.

Enzymatic properties of TVA I-ΔN and TVA II-ΔN mutants

The enzymatic properties of TVA I-ΔN and TVA II-ΔN were different from those of wild-type TVAs. In TVA I-ΔN, the optimum pH changed from 5.0 to 5.5, and the enzyme was unstable under alkaline conditions (Fig. 3(A) a, c). The optimum temperature and thermal stability decreased from 60°C to 50°C (Fig. 3(A) b, d). In TVA II-ΔN, the optimum pH changed from 6.5 to 7.0, and the enzyme was also unstable under alkaline conditions (Fig. 3(B) a, c). The optimum temperature and thermal stability
decreased from 50°C to 40°C (Fig. 3(B) b, d).

Specific activities for various substrates were also observed. The activities of TVA I-ΔN and TVA II-ΔN were drastically decreased, being about 1,500- to 10,000-fold smaller than those of wild-type enzymes. The specific activities for CDs were particularly decreased, namely, that of TVA I-ΔN for γ-CD was about 30,000-fold smaller and those of TVA II-ΔN for α-, β- and γ-CD were about 12,000-fold smaller than that of wild-type TVA I and TVA II, respectively (Table 1).

Table 1. Specific Activities of Wild-type TVAs and TVAs-ΔN Mutants for Various Substrates

<table>
<thead>
<tr>
<th>Substrate</th>
<th>TVA I</th>
<th>TVA I-ΔN</th>
<th>TVA II</th>
<th>TVA II-ΔN</th>
</tr>
</thead>
<tbody>
<tr>
<td>Starch</td>
<td>66.2</td>
<td>7.1</td>
<td>0.011</td>
<td>27.2</td>
</tr>
<tr>
<td>Pullulan</td>
<td>22.1</td>
<td>3.5</td>
<td>0.016</td>
<td>15.2</td>
</tr>
<tr>
<td>α-CD</td>
<td>1.5</td>
<td>0.6</td>
<td>0.042</td>
<td>11.8</td>
</tr>
<tr>
<td>β-CD</td>
<td>2.0</td>
<td>0.9</td>
<td>0.046</td>
<td>15.9</td>
</tr>
<tr>
<td>γ-CD</td>
<td>30.2</td>
<td>1.0</td>
<td>0.003</td>
<td>20.0</td>
</tr>
<tr>
<td>G3</td>
<td>3.4</td>
<td>2.1</td>
<td>0.062</td>
<td>4.4</td>
</tr>
<tr>
<td>G4</td>
<td>17.3</td>
<td>2.4</td>
<td>0.014</td>
<td>28.1</td>
</tr>
<tr>
<td>G5</td>
<td>19.2</td>
<td>3.3</td>
<td>0.017</td>
<td>29.4</td>
</tr>
<tr>
<td>G6</td>
<td>52.3</td>
<td>3.5</td>
<td>0.007</td>
<td>31.3</td>
</tr>
</tbody>
</table>

Oligosaccharides; maltotriose (G3), maltotetraose (G4), maltopentaose (G5) and maltohexaose (G6) were reduced by NaBH₄. The ratio was calculated so that of wild-type TVAs was 100%.

Construction and properties of Y14,16,68A and Y41,82,95A mutants

The results for proteolytic enzymes suggest that domain N in TVA I and TVA II is important for folding, enzymatic activity, and stability. To investigate the relationship between domain N and the enzymatic activities further, we modified several Tyr residues in domain N. Domain N of TVA II contains nine Tyr residues. In this study we focus on Y14, 16, and 68, which are close to the catalytic active site, and Y41, 82, and 95, which are farther from the catalytic active site. The sets of Tyr were replaced with Ala, resulting in Y14,16,68A-TVA II and Y41,82,95-TVA II.

The Y14,16,68A and Y41,82,95A mutants were purified, and the enzymes showed a single band on SDS-PAGE (Fig. 2(C)). Their optimum pH, pH stability, optimum temperature, and thermal stability were measured and these values were identical with those of wild-type TVA II (data not shown).

Kinetic studies of Y14,16,68A and Y41,82,95A mutants

The kinetic parameters of the Y14,16,68A and Y41,82,95A mutants for various substrates were measured. For all substrates, the $k_{cat}$ values of Y14,16,68A and Y41,82,95A were decreased, being 3.1-16% and 3.1-35% of those of wild-type enzyme, respectively. The $K_m$ of these mutants for pullulan were essentially the same as those of wild-type TVA II. However, the $K_m$ of Y14,16,68A for α-CD and maltotriose were 25- and 3-fold larger, and that of Y41,82,95A for soluble starch was 10-fold larger than that of wild-type TVA II, respectively (Table 2). Their $k_{cat}/K_m$ were, therefore, decreased for all substrates, and these values of Y14,16,68A and Y41,82,95A were 0.32-8.6% and 1.9-46% of those of wild-type enzyme, respectively. The decreasing ratios of $k_{cat}/K_m$ of the Y14,16,68A and wild-type enzyme were similar for all the substrates, while those of the
Y41,82,95A and wild-type enzyme for soluble starch, amylose DP 17, and maltotriose were markedly low compared to those for CDs and pullulan. The hydrolysis patterns of Y14,16,68A and Y41,82,95A mutants on starch and pullulan were tested using TLC, and they were identical with those of wild-type TVA II (data not shown).

**Molecular Mass Measurements of TVAs and TVAs-ΔN**

The molecular masses of TVA I and TVA I-ΔN were calculated as 74.0 and 55.2 kDa by amino acid sequence, 71.0 and 59.8 kDa by SDS-PAGE (Fig. 2 (A)), and 75.2 and 57.9 kDa by gel filtration (Fig. 4 (A), 4(B)), respectively. The molecular masses of TVA II and TVA II-ΔN were calculated as 67.5 and 53.7 kDa by amino acid sequence, 65.0 and 55.2 kDa by SDS-PAGE (Fig. 2(B)), and 132.2 and 52.6 kDa by gel filtration (Fig. 4(A), 4(B)), respectively. All the calculated masses of enzymes are shown in Table 3. These results show that TVA I, TVA I-ΔN, and TVA II-ΔN are monomeric structures in solution, while TVA II is a homo-dimeric structure. Thus the domain N of TVA I may be no effect on the subunit conformation, while that of TVA II is important for the formation of the dimeric structure.

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**Table 2.** Kinetic Parameters of Wild-type TVA II, Y14, 16, 68A Mutant and Y41, 82, 95A Mutant for Various Substrates

<table>
<thead>
<tr>
<th>Starch</th>
<th>Pululan</th>
<th>Amylose</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>K_\text{cat} (s^{-1})</strong></td>
<td><strong>K_m (\text{mM})</strong></td>
<td><strong>k_{\text{cat}}/k_m</strong></td>
</tr>
<tr>
<td>Wild-type TVA II</td>
<td>148.4</td>
<td>0.21</td>
</tr>
<tr>
<td>Y14, 16, 68A</td>
<td>5.3</td>
<td>0.32</td>
</tr>
<tr>
<td>Y41, 82, 95A</td>
<td>4.3</td>
<td>1.68</td>
</tr>
<tr>
<td><strong>K_\text{cat} (s^{-1})</strong></td>
<td><strong>K_m (\text{mM})</strong></td>
<td><strong>k_{\text{cat}}/k_m</strong></td>
</tr>
<tr>
<td>Wild-type TVA II</td>
<td>144.4</td>
<td>0.22</td>
</tr>
<tr>
<td>Y14, 16, 68A</td>
<td>10.2</td>
<td>0.18</td>
</tr>
<tr>
<td>Y41, 82, 95A</td>
<td>45.1</td>
<td>0.24</td>
</tr>
<tr>
<td><strong>K_\text{cat} (s^{-1})</strong></td>
<td><strong>K_m (\text{mM})</strong></td>
<td><strong>k_{\text{cat}}/k_m</strong></td>
</tr>
<tr>
<td>Wild-type TVA II</td>
<td>173.8</td>
<td>0.11</td>
</tr>
<tr>
<td>Y14, 16, 68A</td>
<td>5.8</td>
<td>0.16</td>
</tr>
<tr>
<td>Y41, 82, 95A</td>
<td>21.6</td>
<td>0.12</td>
</tr>
</tbody>
</table>

K_m values were calculated using the number-average molecular weights; pullulan, 72,500; amylose (DP = 17), 2,772; maltotriose, 990; maltotetraose, 504; α-CD, 972. K_m values for starch are given as percentages of weight per volume.

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**Fig. 4.** Size Exclusion Chromatography of TVAs, TVAs-ΔN and Size Marker Proteins.

(A) chromatogram of TVAs and TAA. (B) chromatogram of TVAs-ΔN and TAA. The lines are: thin lines, TVA I and TVA I-ΔN; thick lines, TVA II and TVA II-ΔN; broken lines, size marker parotien or TAA. Superose HR12 10/30 column (Pharmacia) was used. The proteins (1.0 mg/ml, 100 μl) were eluted with 50 mM phosphate buffer (pH 6.0) containing 100 mM NaCl, and the flow rate was 0.5 ml/min. The inset shows the line fitting of the elution time vs. logarithm of the molecular mass of size marker proteins, glutamate dehydrogenase: 290 kDa; lactate dehydrogenase: 142 kDa; enolase: 67 kDa; adenylyl kinase: 32 kDa and cytochrome C: 12.4 kDa, and purified TAA as a standard protein were used.
Table 3. Molecular Masses of TVAs and TVAs-A\(\Delta\)N

<table>
<thead>
<tr>
<th></th>
<th>aa Sequence (ratio)</th>
<th>SDS-PAGE (ratio)</th>
<th>Gel Filtration (ratio)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TVA I (kDa)</td>
<td>74.0 (1.00)</td>
<td>71.0 (0.96)</td>
<td>75.2 (1.02)</td>
</tr>
<tr>
<td>TVA I-A(\Delta)N (kDa)</td>
<td>55.2 (1.00)</td>
<td>59.8 (1.04)</td>
<td>57.9 (1.01)</td>
</tr>
<tr>
<td>TVA II (kDa)</td>
<td>67.5 (1.00)</td>
<td>65.5 (0.97)</td>
<td>132.2 (1.98)</td>
</tr>
<tr>
<td>TVA II-A(\Delta)N (kDa)</td>
<td>53.7 (1.00)</td>
<td>55.2 (1.03)</td>
<td>52.6 (0.98)</td>
</tr>
<tr>
<td>TAA (kDa)</td>
<td>52.5 (1.00)</td>
<td>51.0 (0.97)</td>
<td>51.6 (0.98)</td>
</tr>
</tbody>
</table>

Molecular masses were calculated from amino acid sequence, SDS-PAGE, and gel filtration. The ratio was calculated so that of each molecular masses by amino acids sequence were 1. Purified TAA as a standard protein were used.

Discussion

Unlike most other \(\alpha\)-amylases, TVAs efficiently hydrolyze CDs and pullulan as well as starch and maltooligosaccharides, and also have a domain N. To investigate whether domain N is important for the hydrolysis of CDs and pullulan, we constructed domain N deletion mutants, TVA I-A\(\Delta\)N and TVA II-A\(\Delta\)N. The specific activities for not only pullulan and CDs but also starch and maltooligosaccharides were drastically decreased. The values for CDs were particularly low, with those of TVA I-A\(\Delta\)N for \(\gamma\)-CD and TVA II-A\(\Delta\)N for \(\alpha\), \(\beta\), \(\gamma\)-CD being below 0.01% that of wild-type TVA I and TVA II, respectively. The results suggested that domain N is important for the hydrolysis of CDs, and essential for the activities for all the substrates. The enzymatic properties of these mutants were altered and more unstable under alkaline conditions, and their thermal stabilities were 10\(^\circ\)C lower than those of wild-type enzymes. Thus domain N of TVAs is also important for enzymatic activities.

Domain N of TVA II consists of seven \(\beta\)-strands, the first and seventh of which are divided by bulges.\(^9\) Domain N forms a distorted incomplete \(\beta\)-barrel structure, but if one more strand is located in front of the open barrel, the structure is a complete and closed form. Thus many hydrophobic residues in the barrel are located on the exterior to form the solvent accessible surface (Fig. 5(A)). In particular, many Phe residues are located in the barrel. There are reports that aromatic residues, Phe, Tyr, and Trp, of carbohydrate-hydrolyzing enzymes function in substrate recognition and binding.\(^{27,28}\) Other than the Phe residues, domain N is also rich in Tyr residues (Fig. 1(B)). In this study we modified several Tyr residues of TVA II to investigate the relation between domain N and the enzymatic activities. The hydrolytic activities of Y14,16,68A and Y41,82,95A were decreased for all tested substrates. The decreasing ratio of \(k_{\text{cat}}/K_m\) of the Y14,16,68A and wild-type enzyme showed similar trends for all substrates, while those of the Y41,82,95A and wild-type enzyme for soluble starch, amylose, and maltohexaoase were markedly lower than those for CDs and pullulan. These results indicated that domain N plays a significant role in the hydrolysis of not only CDs and pullulan but all substrates.

The results of a preliminary circular dichroism analysis showed that the phase patterns of TVA I and TVA II were different, while those of TVA I-A\(\Delta\)N and TVA II-A\(\Delta\)N were similar (data not shown). Thus both secondary structures of catalytic domain in TVAs-A\(\Delta\)N may be highly conserved. Why do TVAs require domain N for their enzymatic activities? Crystallographic studies have already indicated that the active-site cleft of TVA II is wider and shallower than that of other \(\alpha\)-amylases. For instance, the distance between two Cys of catalytic residues, D325 and D421, in TVA II is 9.6 Å while that of the corresponding residues of \(\alpha\)-amylase from Aspergillus oryzae is 7.8 Å and this wider and shallower cleft may allow TVAs to hydrolyze CDs and pullulan as well as starch. One possible explanation is that this wider and shallower cleft results in less efficient catalytic activities and substrate-recognition, and domain N helps and reinforces the efficiency.

In the family 13 enzymes, Thermus maltogenic amylase,\(^{77}\) Pseudomonas isoaamylyase,\(^{13}\) and Sulfobolus \(\alpha\)-amylase\(^{13}\) also have a domain, called domain N, prior to the N-terminus of domain A/B. Interestingly, the major structure of domain N in these enzymes is also composed of \(\beta\)-strands like TVA II (Fig. 5(B), 5(C)), and the position of domain N in

![Figure 5](image-url)
maltogenic amylase is similar to TVA II, while that of one in others is different. CGTase also has domains other than domains A/B and C. These domains, domains D and E, are located subsequent to the C-terminus of domain C and their positions are not identical with domain N in TVA II, but the major structure of domain E in CGTase, which consists of six β-strands and bulges with many aromatic residues, is similar to TVA II. It has been unclear whether domain N in TVAs binds the substrates, while domain E of CGTase was reported to bind two α-CDs to Tyr and Trp residues (Fig. 5(D)).

Another role of domain N may be to drive the subunit conformation. TVA I, TVA I-ΔN, and TVA II-ΔN form a monomeric structure in solution by the measurement of their molecular masses (Fig. 4(A), 4(B)), while TVA II forms a homo-dimeric structure in solution. Thus domain N of TVA I is no effect on the subunit conformation, while that of TVA II is important for the dimerization. Studies of TVA II and *Thermus* maltogenic amylase, the primary structure of which is highly similar to that of TVA II, indicated that the enzymes are dimeric structures in solution. Kim *et al.* suggested that the protein has an extra sugar-binding space which is formed between domain N of one subunit and domain A/B of the other, and domain N covers in part the top of the active-site cleft. Although it is not clear how the dimeric structure contributes to enzymatic activity, the removal of domain N disrupts the dimeric formation, as may be replacing some amino acids in domain N, and these changes result in loss of activity. To obtain more information on the roles of domain N, we have recently analyzed the structure of TVA II complexed with a substrate-analogue by X-ray analysis, and domain N of TVA II interacted with the substrate-analogue bound at the active center.

In short, these results suggest that domain N of TVA I and TVA II contributes three roles, (1) stabilization for pH and thermal conditions, (2) recognition and hydrolysis for substrates, and (3) dimerization of TVA II molecule.

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