Increasing the hydrophobic interaction between terminal W-motifs enhances the stability of Salmonella typhimurium sialidase. A general strategy for the stabilization of β-propeller protein fold

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Protein engineering of the β-propeller protein aimed at enhancing the structural stability of the protein was carried out using a monomeric single domain β-propeller protein, Salmonella typhimurium sialidase, as a model. Ala53 and Ala69 each located at strands B and C of the W1 motif were mutated to Leu and Val, respectively, to increase the hydrophobic interaction between W1 and W6 motifs. The mutants showed enhanced stability towards guanidine hydrochloride and thermal unfolding. Ala53Leu showed higher stability, probably owing to the capability of the mutated Leu to interact extensively with more residues involved in the hydrophobic interactions between the terminal W-motifs. The mutations, which are located far from the active site, have no significant effect on the enzymatic properties. The strategy to enhance the stability proposed here might be applied to the other β-propeller proteins. Keywords: hydrophobic interaction/β-propeller protein/sialidase/stabilization/terminal W-motifs

Introduction

Since the discovery of the β-propeller structure 17 years ago in the influenza virus neuraminidase (Varghese et al., 1983), this folding motif has rarely been found in other proteins. However, a few years ago the β-propeller structure was found in various proteins elucidated by crystallographic analysis (Oubrie et al., 1999, and references cited therein). Amino acid sequence analyses have also predicted that the structure occurs in many proteins (Springer, 1998; Aravind and Koonin, 1999). The β-propeller structure is composed of a repetitive folding unit called the W-motif, which is arranged circularly like the blade of propeller (Figure 1). The W-motif is an antiparallel β-sheet, which is composed of four β-strands labeled as strands A to D from the inner side to the outer side of the structure. β-Propeller proteins having four to eight W-motifs have been discovered and seven W-motifs are preferred according to the structure model analysis (Murzin, 1992). The diversities of protein functions and number of W-motifs reflect no significant sequence identity among β-propeller proteins. However, the proteins show structural similarities as follows:

1. Terminal regions interaction (‘Velcro’ closure). The N- and C-terminal regions of β-propeller proteins are interacted, closing the peptide in a circular manner. In the β-propeller proteins with five to eight W-motifs, this closure is formed by the interaction of the first and the last β-strands composing the C-terminal W-motif. The closure formed by strands D and C as the first and the last strands, respectively, has been found in the majority of β-propeller proteins. The others are formed by strand B (as the first strand) and C (as the last strand) and by strand C (as the first strand) and B (as the last strand). On the other hand, the closure of β-propeller proteins having four W-motifs is formed by a disulfide-bridge covalent interaction between cysteine residues located before the N-terminal and after the C-terminal W-motifs.

2. Structural scaffold. Superimposition of the backbone between W-motifs in a molecule or against W-motifs from other β-propeller proteins showed a significant similarity especially in the β-strands region, although no sequence identity was observed. This feature suggests that W-motifs have an almost uniform twist angle and the region constructs the structural scaffold of the protein.

3. The position of functional region. Loops, which connect β-strands in W-motifs, are the most varied region in their length and structure. The functional region of most β-propeller proteins is located in the region composed of loops connecting strands B–C and strands D–A rather than in the opposite side composed by loops A–B and C–D. This feature is similar to that of (α/β)8 proteins, known also as TIM-barrel proteins, where a particular side of the proteins composed by loops in the C-terminal of β-strand constructed the functional region (Reardon and Farber, 1995).

The separation of the region responsible for the structural scaffold and functional region, as well as many significant structural similarities demonstrated by β-propeller proteins, provide a basis for protein design using the protein as a model. A secondary structure analysis of membrane-bound pyrroloquinoline quinone (PQQ) glucose dehydrogenase (GDH-A) suggested that the protein consists of two domains, the N-terminal membrane binding domain and C-terminal hydrophilic domain of which the majority is composed of β-sheets (Witarto et al., 1999a). The C-terminal domain was predicted to fold into a β-propeller structure by homology modeling based on the structure of PQQ methanol dehydrogenase, which has eight W-motifs (Cozier and Anthony, 1995). Protein engineering of GDH-A has been conducted with the aim of improving its enzymatic properties suitable for glucose sensor (Yoshida et al., 1999, and references cited therein). Chimeric GDH-As were constructed from the Escherichia coli and Acinetobacter calcoaceticus GDH-As, which show different enzymatic properties. Among the chimeric GDH-As, E97A3, a chimeric protein which has a 97% amino acid sequence from E.coli and the remaining 3% from A.calcoaceticus, was shown to have high thermostability compared with the parental proteins (3- and 12-fold compared with E.coli and A.calcoaceticus GDH-As, respectively) (Sode et al., 1995). Amino acid differences between E97A3 and parental proteins were in the last region of the C-terminal β-propeller domain, part of the last W-motif. Ser771Met mutation was thought to be responsible for the stability. Through further study by mutation to various amino acids of Ser771 of E99A1, a chimeric protein having an amino acid sequence similar to that of E97A3 except for residue 771 but lower stability compared with E.coli GDH-A
suggested that hydrophobic interaction may govern the thermal stability of the chimeric protein (Witarto et al., 1999b). According to the model structure (Cozier and Anthony, 1995), the side chain of residue 771 at the C-terminal W-motif faces the N-terminal W-motif. Hence the interaction between the terminals W-motifs may be important in maintaining the structure of the β-propeller protein.

However, further study using the β-propeller protein with an elucidated tertiary structure is required in order to verify the strategy of stabilization. Here, the protein engineering of sialidase from *Salmonella typhimurium* (Figure 1) is reported with the aim of proposing a strategy to stabilize the β-propeller protein. *S. typhimurium* sialidase is a β-propeller protein having six W-motifs which does not require any cofactors for stability or activity. Furthermore, the protein is a monomeric single domain β-propeller protein with a high resolution of tertiary structure information available (Crennel et al., 1993). Hence *S. typhimurium* sialidase would be a good model for the study of the β-propeller protein.

**Materials and methods**

**Gene cloning and mutagenesis**

Sialidase gene, *nanH*, was cloned from the *S. typhimurium* IFO12529 genome by polymerase chain reaction (PCR) using primers designed according to the published sequence deposited at Gen-Bank (http://www.ncbi.nlm.nih.gov/) with accession number M55342 (Hoyer et al., 1992). NcoI and HindIII sites were incorporated into the forward (5’-GGCCATGGCTGTA-GAAAATCCGTGGTTTTTAAAGCT-3’) and reverse primers (5’-GGGAAAGCTTTTAATTGTATGTTTTACTGGTAA-3’), respectively (restriction enzyme sites are underlined). The introduction of the NcoI site resulted in the substitution of the second amino acid residue from Thr to Ala. The gene was inserted into the multi-cloning site of the expression vector pTrc99A (Pharmacia, Sweden). The resulting plasmid designated pNanH was transformed into *E. coli* DH5α MCR, which is used as the host strain for the expression of sialidase. Site-directed mutagenesis was performed according to the ODA method (Hashimoto-Gotoh et al., 1995), using the gene fragment obtained by *KpnI*–*HindIII* digestion of *nanH*. The fragment was inserted into the plasmid pKF18K (Takara, Japan) digested with the same enzymes. Synthesized primers, 5’-ATCAATGTTATTTCTTGTGAGGGCGCCCATACAC-3’ and 5’-CTTTATATGACACAGTACCGGCGCTCCGTAGCAC-3’ were used for the construction of mutants Ala53Leu and Ala69Val, respectively (mutated codons are underlined). *E. coli* BMHS81-18mutS and *E. coli* MV1184 (Takara) were used for constructing the mutants, performed according to the instruction manual. The sequence of *nanH* and introduction of mutations were confirmed by gene sequencing using ABI-310 DNA sequencer (Perkin-Elmer, USA).

**Enzyme production, purification and assay**

*E. coli* DH5α MCR/pNanH was cultured in 7 l of Luria–Bertani medium using a jar-fermenter. When the cell growth monitored by measuring OD660 nm was at the middle of exponential growth (around OD660 nm = 2), an enzyme expression was induced by the addition of 0.3 mM IPTG. This was followed by shifting the temperature of the medium from 37 to 30°C. After cell growth had reached the stationary phase (~6 h from the beginning of cultivation), cells were collected by centrifugation. Purification of sialidase was carried out according to the published method (Hoyer et al., 1991) with several modifications as follows. Cells were washed with water containing 0.75% NaCl, then suspended with 10 mM potassium phosphate buffer, pH 7.0 (buffer A). The suspension was subjected to French pressure to disrupt the cells, which were then ultracentrifuged (160 500 g, 1.5 h, 4°C) to obtain the soluble fraction. After overnight dialysis against buffer A, the soluble fraction was poured into DEAE-Toyopearl 650M beads (Tosoh, Japan) previously equilibrated with buffer A. The enzyme was contained in the unbound fraction, which was separated from the beads by centrifugation. Then the fraction was applied to a cation-exchange column of CM-Toyopearl 650M (20 cm × 16 mm i.d.) (Tosoh). After washing the column with five bed-volumes of buffer A, the enzyme was eluted by a linear gradient of 180 ml of buffer A containing 0.2 M NaCl for 1 h. Active fractions were pooled and dialyzed overnight against buffer A. All purification steps were performed at 4°C. Enzyme purity was checked by SDS–PAGE on a gradient gel, PhastGel gradient 8–25 (Pharmacia) and silver stained using a PhastSystem electrophoresis apparatus (Pharmacia). An enzyme assay was carried out using a synthetic substrate, 2-ON-(p-nitrophenyl)-N-acetyl-α-D-neuraminic acid (Seikagaku, Japan) at 25°C in 10 mM potassium phosphate buffer, pH 7.0. Enzyme activity was measured by the increase in absorbance at 420 nm originated from the liberated p-nitrophenol. One unit was defined as the activity of enzyme to catalyze the hydrolysis of 1 μmol/min of neuraminic acid at 25°C.

**Circular dichroism (CD)**

Far-UV CD spectra were measured with a J-720 spectropolarimeter (JASCO, Japan) between 260 and 190 nm at room temperature using a circular quartz cell with a pathlength of 0.1 cm (JASCO). Protein concentrations were 200 μg/ml. CD spectra were averaged from three scans, which were then subtracted by the buffer baseline. The secondary structure contents were estimated using the Convex Constraint Analysis (CCA) program (Perzel et al., 1991) with a database of 23 soluble protein CD spectra as reference (Perzel et al., 1992). Thermal denaturation was performed by increasing the temperature from 25 to 90°C with a temperature slope of 50°C/h using a rectangular quartz cell having a pathlength of 0.1 cm (GL Sciences, Japan). Ellipticity was measured at 197 nm with 0.2°C resolution, 4 s response time and 1.0 nm bandwidth.

**Thermal inactivation**

Thermal inactivation was measured by incubating 196 μl of the enzyme solution with a protein concentration of 10 μg/ml at each temperature for 10 min. Samples were then immediately
stored on ice for 1 min to stop the denaturation reaction, which was followed by equilibration at room temperature for 10 min. A 4 µl volume of the substrate solution (final concentration, 0.1 mM) was added to the sample and after vortex mixing, the absorbance increase at 420 nm was measured to determine the residual enzymatic activity.

**Guanidine hydrochloride (GdnHCl) unfolding**

Unfolding was carried out by incubating 500 µl sample solutions (protein concentration, 30 µg/ml) containing various concentrations of GdnHCl at 25°C in a water-bath for 12 h. Fluorescence spectra of the samples were then measured using an RF-5000 spectrofluorimeter (Shimadzu, Japan) by scanning the emission wavelength from 295 to 500 nm after excitation at 280 nm. Fluorescence data at 344 nm were used for the unfolding analysis. Molecular biology grade GdnHCl (Kanto Chemicals, Japan) was used for the unfolding experiments. Data analysis was performed assuming a two-state unfolding process according to a published method (Pace and Scholtz, 1997) and obtained theoretical curves.

**Molecular modeling**

Molecular modeling of the mutant enzymes was performed using the InsightII molecular modeling package (Biosym, USA). The coordinates of sialidase, which was deposited in the PDB with accession code 2SIM.PDB (Crennel et al., 1993), were used as a template.

**Results**

**Amino acid sequence**

The published nucleotide sequence of *S.typhimurium* LT2 sialidase (Hoyer et al., 1992) lacks an insertion of an adenine residue at a position around 1060 of the structural gene *nanH*. The revised nucleotide sequence is deposited in the GenBank under the accession number M55342. A crystallographic analysis of sialidase from the same strain showed that residue 329 is an aspartic acid (Crennel et al., 1993), while the amino acid sequence deduced from the revised nucleotide sequence suggested an alanine residue (Hoyer et al., 1992). Sequencing of the sialidase gene from *S.typhimurium* IFO12529 revealed an adenine residue at the second codon of the residue 329, instead of cytosine which results in the mutation of alanine to aspartic acid as observed in the crystal structure of *S.typhimurium* LT2. The other amino acid sequence was identical with the revised sequence of *S.typhimurium* LT2 except for the Thr2Ala mutation resulting from the introduction of the Ncol restriction site for the cloning of the sialidase gene.

**Production of recombinant sialidases**

*S.typhimurium* sialidase is expressed in the cytoplasm since it does not have a signal peptide (Hoyer et al., 1991, 1992). The protein is a basic protein, so after removing most of the acidic proteins using anion-exchange beads, the protein was purified using a CM-Toyopearl 650M cation-exchange column. Using the purification procedure reported here, purified sialidase (Figure 2) could be obtained in shorter steps compared with the published procedure which included size-exclusion chromatography in the last step (Hoyer et al., 1991). An average of 13 mg of purified protein was obtained from 1 l of cultivation medium. Similar results were obtained with the mutant enzymes.

**Circular dichroism spectra**

The far-UV CD spectrum of wild-type sialidase showed a typical β-sheet protein with a single minimum and maximum around 217 and 194 nm, respectively (Figure 3). A secondary structure estimation using the CCA program (Perczel et al., 1991, 1992) predicted a majority of β-sheet content (β-sheet, 45%; α-helix, 6.0%; turn, 9.0%; random, 40.0%) in accordance with the elucidated β-propeller structure. CD spectra of mutant enzymes showed a very similar pattern, suggesting that no significant structural changes had occurred as a result of mutations.

**Thermal stability**

The thermal stability of sialidases was studied by monitoring residual enzymatic activities after heat treatment at each temperature. As shown in Figure 4, both mutants showed increased thermal stability with Ala53Leu acquiring the highest stability. To study the stability of the polypeptide structure, thermal denaturation was monitored by CD using the signal at 217 nm where the largest changes were observed at the measurable wavelength (Figure 5). Similarly to the results observed for residual activity, the mutants showed stability decreasing in the order Ala53Leu > Ala69Val > wild-type (Table I).

**Guanidine hydrochloride (GdnHCl) unfolding**

GdnHCl unfolding was carried out to study the stability of sialidases towards chemical denaturation and to obtain the thermodynamic parameters with reversible denaturation (Pace and Scholtz, 1997). The unfolding was studied by fluorescence...
Fig. 4. Thermal inactivation of wild-type and mutant sialidases. Residual activity was measured at room temperature after 10 min of incubation at each temperature. Open circles, closed squares and closed circles represent wild-type, Ala69Val and Ala53Leu, respectively.

Fig. 5. Thermal denaturation of wild-type and mutant sialidases as measured by CD. Thermal denaturation was measured by monitoring the ellipticity at 197 nm. Data from 25 to 40°C were removed for clarity. From left to right wild-type, Ala69Val and Ala53Leu.

Fig. 6. Fluorescence spectra of the unfolded wild-type sialidase. Spectra from various concentrations of denaturant are shown. Excitation was performed at 280 nm and the spectra were subtracted from the buffer baselines.

Table I. Thermodynamic parameters of wild-type and mutant sialidases

<table>
<thead>
<tr>
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<th>(T_m) (°C)\footnote{(T_m) values were obtained from thermal denaturation measured by CD at 217 nm.}</th>
<th>[GdnHCl] (M)</th>
<th>Δ(G(H_2O)) at 25°C (kcal/mol)</th>
<th>ΔΔ(G) (kcal/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>57</td>
<td>1.33</td>
<td>4.24</td>
<td>–</td>
</tr>
<tr>
<td>Ala69Val</td>
<td>60</td>
<td>1.42</td>
<td>4.86</td>
<td>0.62</td>
</tr>
<tr>
<td>Ala53Leu</td>
<td>62</td>
<td>1.64</td>
<td>5.05</td>
<td>0.81</td>
</tr>
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</table>

spectroscopy. Sialidase is composed of 342 amino acid residues with six tryptophan residues. Two Trp residues are located each at W1 and W4 motifs while the other four Trp residues are clustered in W3 motif. Exciting wild-type sialidase at 280 nm resulted in a fluorescence spectrum with a peak at 344 nm (Figure 6). Unfolding of the protein resulted in a gradual decrease in intensity at 344 nm without significant red shifting of the wavelength peak. Similar results were obtained with the mutant enzymes. The decrease in intensity at 344 nm was used to monitor the unfolding of sialidases. Diluting the unfolded protein resulted in a recovery of ~80% of the initial peak intensity. Dialysis of the unfolded protein resulted in a recovery of 90% of the initial activity. These results suggested that the GdnHCl unfolding of sialidases is a reversible process. As shown in Figure 7, both mutants unfolded at higher concentrations of GdnHCl in the same order as observed for thermal stability. Calculations of the thermodynamic parameters from the unfolding data assuming a two-state denaturation process (Pace and Scholtz, 1997) estimated that wild-type sialidase has a Δ\(G(H_2O)\) at 25°C of ~4.2 kcal/mol, whereas Ala53Leu has a 0.8 kcal/mol higher stability (Table I). There is very limited data available on the Δ\(G(H_2O)\) of β-sheet proteins which could be used for comparison. However, the data obtained here are lower than the average Δ\(G(H_2O)\) of protein with a mixture of secondary structures of the similar size (Pace and Scholtz, 1997).
Enzyme activity

The mutations carried out in this study were positioned in the scaffold region of the β-propeller protein, which is far from the active site. Thus, it was expected that the mutations would not affect the enzymatic activities of the enzyme. Table II shows the $K_{\text{m}}$, $V_{\text{max}}$, and $V_{\text{max}}/K_{\text{m}}$ values for the enzymes obtained using a synthetic substrate, 2-O-(p-nitrophenyl)-N-acetyl-α-D-neuraminic acid. As expected, the mutants and wild-type sialidases showed almost identical enzymatic activities.

Discussion

The β-propeller structure has been found in many proteins having various functions (Oubrie et al., 1999, and references cited therein). β-Propeller proteins functioning as enzymes, such as PQQ glucose dehydrogenases, galactose oxidase, etc., are expected as components of biosensors (Schimdt, 1997). This application used to require high protein stability. On the other hand, the separation of the functional region and the region responsible for the structural scaffold suggested that the protein is a good model for protein design (i.e. altering structural properties while maintaining functional region and vice versa). In this paper, the protein engineering of the β-propeller protein focusing on structural stability using S. typhimurium sialidase as a model was reported. This protein was chosen since it is a monomeric and a single domain β-propeller protein with tertiary structure information available at high resolution (Crennel et al., 1993).

Sialidase has been produced and purified by a simple procedure in a sufficient quantity for the structural study. Guanidine hydrochloride (GdnHCl) unfolding of sialidase showed reversible denaturation of this all-β-sheet protein. This suggested that the protein, which does not have any cofactor, is suitable also as a model for the study of kinetic unfolding of all β-sheet proteins about which there is still a lack of knowledge compared with other types of protein (Baldwin, 1993).

Crystallographic analysis of β-propeller proteins suggested several stabilization schemes of the proteins. Tryptophan residues of strand D are known to stack with glycine residues of strand D from the adjacent W-motif in PQQ methanol dehydrogenase (Xia et al., 1996). A hydrogen-bonding tetrad comprising of aspartic acid–histidine–serine/threonine–tryptophan connecting strands in a W-motif was observed in the G-protein β-subunit (Wall et al., 1995; Sondek et al., 1996). A salt bridge was maintained between arginine/lysine of strand C and aspartic acid/glutamic acid in the CD loop of the next W-motif in the soluble PQQ glucose dehydrogenase (GDH-B) (Oubrie et al., 1999). These interactions were conserved among the W-motifs of the proteins and thus suggested a stabilizing role of the interactions. However, these interactions were limited to specific β-propeller proteins and no further study has been reported on the role of interactions. Although sequence identity has not been found among β-propeller proteins, hydrophobic residues have been observed to be frequently conserved in the β-strands of W-motifs. These hydrophobic residues are involved in the hydrophobic interaction between W-motifs. Thus, a strategy focusing on this interaction is thought to be applicable to all β-propeller proteins. Previous studies of membrane-bound PQQ glucose dehydrogenase (GDH-A) suggested that interactions of terminal regions are important in maintaining the overall β-propeller structure (Witarto et al., 1999b). The interactions of terminal regions include the interaction of the first and the last strand in the last W-motif (‘Velcro’ closure) and the hydrophobic interaction between N- and C-terminal W-motifs, both of which are conserved in all β-propeller proteins. Here, the study was focused on the hydrophobic interaction between W-motifs.

Hydrophobic interaction as a cluster of hydrophobic residues has been observed between the W-motifs of S. typhimurium sialidase, a β-propeller protein with six W-motifs (Crennel et al., 1993). The alanine residue in the W1-motif was not involved in the hydrophobic interaction between W1- and W6-motifs (Figure 8A). Mutations of these residues into more hydrophobic residues were carried out to increase the hydrophobic interaction. Molecular modeling of the mutant enzymes predicted that mutation of Ala53 to Leu (Figure 8B) and Ala69 to Val resulted in more residues being involved in the hydrophobic interaction, as confirmed by the wild-type, as summarized in Table III. The extensive interaction of Ala53Leu may be responsible for the higher stability. Furthermore, since the mutations were located in the scaffold region, which was separated from the functional region, the mutants were shown to have had no significant enzymatic activities altered. Therefore, the proposed strategy for the stabilization of the β-propeller protein conformation may be a ‘generic’ strategy applicable to other β-propeller proteins.

### Table II. Enzymatic properties of wild-type and mutant sialidases

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<thead>
<tr>
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<th>$K_{\text{m}}$ (mM)</th>
<th>$V_{\text{max}}$ (U/mg protein)</th>
<th>$V_{\text{max}}/K_{\text{m}}$</th>
</tr>
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<tr>
<td>Wild-type</td>
<td>0.086</td>
<td>120</td>
<td>1395</td>
</tr>
<tr>
<td>Ala69Val</td>
<td>0.080</td>
<td>115</td>
<td>1437</td>
</tr>
<tr>
<td>Ala53Leu</td>
<td>0.090</td>
<td>112</td>
<td>1244</td>
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Enzyme assays were carried out using the synthetic substrate 2-O-(p-nitrophenyl)-N-acetyl-α-D-neuraminic acid at 25°C in 10 mM potassium phosphate buffer, pH 7.0.

### Table III. Residues involved in the hydrophobic interactions at positions 53 and 69

<table>
<thead>
<tr>
<th>Residue 53</th>
<th>Phe9: Cε2, Cε2</th>
<th>Phe9 : Cγ, Cδ2, Cε2, Cγε1</th>
</tr>
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<tbody>
<tr>
<td>Wild-type</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mutants</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Leu53</td>
<td>Phe9 : Cγε1</td>
<td></td>
</tr>
<tr>
<td>Val69</td>
<td>Phe9 : Cγε1</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Residue 69</th>
<th>Phe9: Cε1, Cε1</th>
<th>Phe9: Cε1, Cε1</th>
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<tbody>
<tr>
<td>Wild-type</td>
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<tr>
<td>Leu53</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Val69</td>
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Fig. 8. Structure surrounding residue 53 of the wild-type (A) and Ala53Leu (B). Residues involved in the hydrophobic interaction were picked up.
References


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