How Oligomerization Contributes to the Thermostability of an Archaeon Protein

PROTEIN L-ISOASPARTYL-O-METHYLTRANSFERASE FROM SULFOLOBUS TOKODAII

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To study how oligomerization may contribute to the thermostability of archaeon proteins, we focused on a hexameric protein, protein L-isoaspartyl-O-methyltransferase (PIMT) from Sulfolobus tokodaii (StoPIMT). The crystal structure shows that StoPIMT has a distinctive hexameric structure composed of monomers consisting of two domains: an S-adenosylmethionine-dependent methyltransferase fold domain and a C-terminal α-helical domain. The hexameric structure includes three interfacial contact regions: major, minor, and coiled-coil. Several C-terminal deletion mutants were constructed and characterized. The hexameric structure and thermostability were retained when the C-terminal α-helical domain (Tyr206–Thr231) was deleted, suggesting that oligomerization via coiled-coil association using the C-terminal α-helical domains did not contribute critically to hexamerization or to the increased thermostability of the protein. Deletion of three additional residues located in the major contact region, Tyr203–Asp204–Asp205, led to a significant decrease in hexamer stability and chemico/thermostability. Although replacement of Thr146 and Asp204, which form two hydrogen bonds in the interface in the major contact region, with Ala did not affect hexamer formation, these mutations led to a significant decrease in thermostability, suggesting that two residues in the major contact region make significant contributions to the increase in stability of the protein via hexamerization. These results suggest that cooperative hexamerization occurs via interactions of “hot spot” residues and that a couple of interfacial hot spot residues are responsible for enhancing thermostability via oligomerization.

Thermophilic organisms grow optimally at temperatures of >70 °C. The proteins isolated from these organisms are usually more thermostable than homologous proteins from mesophiles, despite the fact that they have similar three-dimensional structures and identical catalytic mechanisms to the mesophilic proteins as well as sequence homologies of 40–85% (1). Extensive studies have focused on identifying key determinants or common factors responsible for the thermostability of thermophile proteins, and the following structural features have been proposed as responsible for the high thermostability: decreased solvent-exposed surface area (2), increased polar interactions at the molecular surface (3–10), higher packing density within the protein (10–12), greater core hydrophobicity (10, 13–15), shorter surface loops (11, 16), and more hydrogen bonds (7, 10, 17–19) relative to those of ordinary proteins as well as oligomerization (10, 20–25).

The protein L-isoaspartyl-O-methyltransferase (PIMT; EC 2.1.1.77) functions as an enzyme in the repair of age-damaged proteins in which asparagines and aspartates have been spontaneously deamidized and isomerized into L-isoaspartyl residues; it catalyzes S-adenosylmethionine (AdoMet)-dependent methylation of the α-carboxyl group of the L-isoaspartyl residues to form 1-iso-Asp-α-methyl ester (26, 27). Crystal structures of PIMT have been reported for both mesophiles and thermophiles (Thermotoga maritima (28), Pyrococcus furiosus (29), humans (30, 31), and Drosophilia melanogaster (32)), revealing similar three-dimensional structures. The structures include an AdoMet-dependent methyltransferase fold, which is a modified Rossmann fold consisting of a central seven-stranded β-sheet flanked by α-helices on both sides; this structure is common to the entire AdoMet-dependent methyltransferase family, including those having DNA, RNA, proteins, polysaccharides, lipids, and small molecules as substrates for methyltransferase reactions (33). We have succeeded in clarifying the functional and structural properties of PIMT from the thermophilic archaeon Sulfolobus tokodaii (StoPIMT), whose optimal growth temperature is 80 °C, and have found a unique hexa-

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The atomic coordinates and structure factors (code 1bgb) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/)

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1 The abbreviations used are: PIMT, protein L-isoaspartyl-O-methyltransferase; StoPIMT, PIMT from S. tokodaii; StoPIMT-dxx, a series of deletion mutants of StoPIMT containing only the sequence from Met1 to amino acid number xxx; StoPIMT-WT-mutant, a StoPIMT double mutant in which Thr146 and Asp204 are both replaced with Ala; StoPIMT-d205-mutant, a StoPIMT-d205 double mutant in which Thr146 and Asp204 are replaced with Ala; GdnHCl, guanidine hydrochloride; SEC, size exclusion chromatography; DSC, differential scanning calorimetry; PfPIMT, PIMT from Pyrococcus furiosus; AdoMet, S-adenosylmethionine; AdoHcy, S-adenosyl-l-homocysteine; MBP, maltose-binding protein.
meric structure that was not previously reported in the AdoMet-dependent methyltransferase family.

It has been proposed that oligomerization is critically important for the stability of archaen proteins (10, 20–25), and recent structural studies have demonstrated that many archaen proteins have a homo-oligomeric structure. Some reports have shown correlations between oligomerization and the hyperthermostability of archaen proteins (20, 21). Oligomerization is usually through interfacial interaction (34–36), in which subunits cooperatively interact with each other in several ways (e.g. domain swapping and coiled-coil association).

In this report, we first describe the crystal structure of StoPIMT at 2.8 Å resolution. The monomeric structure of StoPIMT consists of two domains, an AdoMet-dependent methyltransferase fold domain and a distinctive C-terminal α-helical domain. Six monomers associate into a hexamer, in which there are three contact regions per monomer, referred to as the major, minor, and C-terminal α-helical contact regions. We investigated whether and how oligomerization is correlated with enhancement of thermostability by constructing several deletion mutants; our results suggest the existence of “hot spot” residues in the subunit interfaces that function in cooperative oligomerization and in enhancing thermostability via oligomerization. On the basis of these observations, we discuss how oligomerization contributes to the thermostability of this protein.

**EXPERIMENTAL PROCEDURES**

**Materials**—All enzymes used in genetic engineering were obtained from Takara Shuzo (Kyoto, Japan), Toyobo (Osaka, Japan), and New England Biolabs (Beverly, MA). Isopropyl-1-thio-β-D-galactopyranoside was obtained from Wako Fine Chemicals Inc. (Osaka, Japan). All other reagents were of biochemical research grade.

**Construction of Expression Vectors for StoPIMT, Various Deletion Mutants, and Mutated StoPIMT**—The gene encoding StoPIMT was amplified by KOD-Plus DNA polymerase (Toyobo), using S. tokodaii genomic DNA as a template and designed primers as follows. NcoI and BamHI sites were incorporated into the ST1123-reverse (5'-NNGNCCATGGCCAGTTAAAGAAAGCTTTATGAGAGAAG-3') and ST1123-forward (5'-NNGNAGATCTTTAATTCTTCTTTAATTATCTAATATTTG-3') sequences, respectively (restriction enzyme sites are underlined, and mutated sites are in italics). ST1123-D204A-forward was 5'-AGACCTCCAATTCTTCCGAACATACTTC-3', ST1123-D204A-reverse, ST1123-forward, and the four synthesized primers were used (restriction enzyme sites are underlined, and mutated sites are in italics). ST1123-D205, was achieved by PCR mutagenesis. For the resultant electron density map was of sufficient quality to rebuild the monomeric structure of StoPIMT, some restraints were used, and the restraint weight was gradually decreased as the maximum density level using the procedure in the program CNS (47). First we performed multiwavelength anomalous diffraction phasing, three wavelengths were chosen of the energy spectrum of the synchrotron radiation at 1.2 Å, corresponding to the maximum f'′ value (peak, 0.9793 Å), minimum f′ (edge, 0.9795 Å), and the reference point (remote, 0.9790 Å). The three-wavelength diffraction data set was collected to a resolution of 2.8 Å with a MAR Research CCD detector. The crystal of selenomethionine-substituted StoPIMT belongs to space group P23 with unit cell dimensions of a = b = c = 277.90 Å. The data were indexed and integrated with the program MOSFLM (41) and were further scaled and merged with the program SCALA in the CCP4 package (42).

**X-ray Diffractions**—X-ray diffraction of selenomethionine-substituted StoPIMT was performed on the beamline BL44XU at SPring-8 (Harima, Japan), under cryogenic conditions (100 K). For multiwavelength anomalous diffraction phasing, three wavelengths were chosen of the energy spectrum of the synchrotron radiation at 1.2 Å, corresponding to the maximum f′′ value (peak, 0.9793 Å), minimum f′ (edge, 0.9795 Å), and the reference point (remote, 0.9790 Å). The three-wavelength diffraction data set was collected to a resolution of 2.8 Å with a MAR Research CCD detector. The crystal of selenomethionine-substituted StoPIMT belongs to space group P23 with unit cell dimensions of a = b = c = 277.90 Å. The data were indexed and integrated with the program MOSFLM (41) and were further scaled and merged with the program SCALA in the CCP4 package (42).

**Structure Solution and Refinement**—The initial phase and subsequent phase improvement were done with the program SOLVE/RESCALE (43–45). First we performed multiwavelength anomalous diffraction phasing, three wavelengths were chosen of the energy spectrum of the synchrotron radiation at 1.2 Å, corresponding to the maximum f′′ value (peak, 0.9793 Å), minimum f′ (edge, 0.9795 Å), and the reference point (remote, 0.9790 Å). The three-wavelength diffraction data set was collected to a resolution of 2.8 Å with a MAR Research CCD detector. The crystal of selenomethionine-substituted StoPIMT belongs to space group P23 with unit cell dimensions of a = b = c = 277.90 Å. The data were indexed and integrated with the program MOSFLM (41) and were further scaled and merged with the program SCALA in the CCP4 package (42).

**Positional and individual B factor refinement was carried out with the program CNS (47), using reflections ranging from 15 to 2.8 Å. A random 10% of all observed reflections were set aside for cross-validation analysis and were used to monitor throughout the refinement by calculating the free R value (Rfree). Noncrystallographic symmetry restraints were used, and the maximum likelihood method was used as model refinement progressed. The water molecules were located by peak searching on the SIGMAA-weighted mF - DF, map, and some water molecules, which occupied irrelevant positions, were deleted on the basis of their real space correlation coefficient and maximum density level using the procedure in the program CNS (47). Finally, we obtained the crystallographic parameters and refinement statistics are summarized in Table I.
**Table 1**

| Parameter                      | Se-StoPIMT |
|-------------------------------|------------|
| Space group                   | F23        |
| Cell dimensions (Å)           | a = b = c = 277.2 |
| Beamline                      | BL4XU      |
| Resolution (Å<sup>2</sup>)    | 20 to 2.8 (2.95 to 2.8) |
| Wavelength (Å)                | 0.9793     |
| R<sub>rms</sub> (%)           | 8.2 (35.7) |
| Completeness (%)<sup>p</sup>  | 100.0 (100.0) |
| Observed reflections          | 988,964    |
| Unique reflections            | 43,276     |
| I<sub>o</sub> (I)             | 7.9        |
| Multiplicity                  | 22.9       |
| Refinement and model quality  | 15 to 2.8  |
| Resolution range (Å)          | 23,796     |
| No. of reflections            | 4.5        |
| F/|F<sub>o</sub>| cut-off (%) | 6.6          |
| R factor<sup>2</sup>          | 0.198      |
| R<sub>free</sub> factor<sup>2</sup> | 0.245     |
| Total protein atoms           | 7172       |
| Total water atoms             | 429        |
| Average B factor (Å<sup>2</sup>) | 36.8       |

<sup>a</sup> The values in parentheses refer to data in the highest resolution shell.
<sup>b</sup> R<sub>rms</sub> = \( \sum_{h,i} |F_{o,h,i} - F_{c,h,i}|/\sum_{h,i} |F_{o,h,i}| \), where |I<sub>o</sub>| represents the mean intensity of a set of equivalent reflections.
<sup>c</sup> R factor = \( \sum_{h,i} |F_{o,h,i} - F_{c,h,i}|/\sum_{h,i} |F_{o,h,i}| \), where F<sub>o</sub> and F<sub>c</sub> are the observed and calculated structure factor amplitudes.
<sup>d</sup> The R<sub>free</sub> factor was calculated for the R factor by using a random 10% subset of all reflections.

**Size Exclusion Chromatographic Analyses of StoPIMT and Its Mutants**—Size exclusion chromatography using a HiLoad Superdex 200-7.5 cm column (16 × 300 mm; Amersham Biosciences) was carried out for analysis of the extent of oligomerization of StoPIMT and its mutants. The column was equilibrated with 50 mM Tris-HCl, pH 8.0, containing 200 mM NaCl, and then 2 ml of purified protein was applied to the column at a flow rate of 2.5 ml min<sup>-1</sup>.

**Differential Scanning Calorimetry (DSC)**—All DSC measurements were carried out with a nanoDSC II calorimeter (Calorimetry Science Corp.). Proteins were dialyzed against phosphate-buffered saline buffer (125 mM phosphate buffer, pH 7.0, containing 150 mM NaCl). The dialysis buffer was used as a reference solution for the DSC scan. Protein samples of 1.2–1.7 mg ml<sup>-1</sup> were heated from 0 to 125 °C at a scanning rate of 1 K min<sup>-1</sup>.

**CD Spectrometry**—To compare the tolerance to GdnHCl of StoPIMT and its mutants, purified and concentrated proteins were diluted in 50 mM Tris-HCl (pH 8.0) buffer containing various concentrations of GdnHCl (0–8 M). After 24 h, CD spectra were measured in an AVIV circular dichroism spectrometer (Proteinier Co.): path length, 1.0 mm; resolution, 0.2 mm; average time, 4 s. The results are expressed as the mean residue ellipticity ([θ]<sub>θ</sub>), and [θ]<sub>θ</sub> values at 222 nm were plotted as a function of GdnHCl concentration.

**RESULTS**

**Crystal Structure of StoPIMT**—The crystal structure of StoPIMT was determined at 2.8 Å resolution by the single-wavelength anomalous dispersion method (Table I). The asymmetric unit contains four StoPIMT monomers with a Matthews’ coefficient (V<sub>M</sub>) of 4.21 Å<sup>3</sup> Da<sup>-1</sup> and a solvent content of 70.6%. The monomeric structure was composed of seven β-strands (β<sub>1</sub>–β<sub>7</sub>) and eight α-helices (α<sub>1</sub>–α<sub>8</sub>), forming two domains: the AdoMet-dependent methyltransferase fold domain (blue to gold; Fig. 1A), which is common to all of the enzymes in this family (33), and a characteristic C-terminal α-helical domain (red; Fig. 1A).

The former is described as a modified Rossmann fold consisting of a central seven-stranded β-sheet (β<sub>1</sub>–β<sub>7</sub>) flanked by α-helices (α<sub>1</sub>–α<sub>8</sub>) on both sides. In contrast to the other AdoMet-dependent methyltransferases from various species whose crystal structures have been published (28–32), in all PIMT proteins the two strands β<sub>6</sub> and β<sub>7</sub> are exchanged relative to the arrangement of the Rossmann fold (28–32). The structure of StoPIMT reported here also included this rearrangement of two β-strands (Fig. 1B), and therefore the StoPIMT structure can be superimposed well on the other PIMT structures from T. maritima, P. furiosus, humans, and Drosophila; the respective root mean square differences for superposition are 1.52 Å for 175 Ca atoms, 1.23 Å for 190 Ca atoms, 1.39 Å for 170 Ca atoms, and 1.42 Å for 163 Ca atoms.

The C-terminal α-helical domain, which consists of a long loop and one α-helix (α<sub>9</sub>), protrudes from the methyltransferase fold, and the three α<sub>9</sub>-helices in three separate monomers related by the crystallographic 3-fold symmetry axis form a unique coiled-coil assembly (Fig. 1D; see “Hexameric Assembly of StoPIMT”). Such a coiled-coil assembly has not been identified in any other solved AdoMet-dependent methyltransferase. We should also note that the C-terminal 30 residues corresponding to this domain show no significant sequence similarity to any sequence in the Swiss-Prot/TrEMBL data base (available on the World Wide Web at ca.expasy.org/).

In the structure of P. furiosus PIMT, very clear electron density corresponding to the cofactor S-adenosyl-l-homocysteine (AdoHcy) was recognized (29). In the present structure of StoPIMT, the F<sub>o</sub> – F<sub>c</sub> electron density map indicated that an AdoHcy molecule was bound to two of four molecules in the asymmetric unit (chains A and C in Fig. 1C). AdoHcy was associated with Thr<sup>154</sup> O<sub>y</sub>, Gly<sup>197</sup> O<sub>y</sub>, Glu<sup>199</sup> O<sub>e</sub>, Gly<sup>197</sup> O<sub>e</sub>, and Gly<sup>125</sup> N<sub>e</sub>, suggesting that the recognition manner seems to be identical to that of other PIMTs. As with P. furiosus PIMT (PfuPIMT), this was an unexpected result, because no cofactor was added in the purification and/or crystallization steps for either protein. The bound AdoHcy is presumably derived from PIMT activity during the growth of the E. coli cells, and the enzyme must have retained the cofactor through all of the experimental procedures.

**Hexameric Assembly of StoPIMT**—The PIMTs reported so far function as monomers, and there has been no report of their oligomerization. In contrast, the crystal structure of StoPIMT reveals that it forms a hexamer (Figs. 1, C and D), which is consistent with the results of size exclusion chromatography (Fig. 1E).

We now describe the elaborate hexameric assembly of this enzyme. The four monomers in the asymmetric unit create two disulfide-bridged dimers. Two disulfide linkages are formed between chains A and B and between chains C and D (Fig. 1C), using the identical cysteine residue Cys<sup>149</sup>. Interestingly, this Cys<sup>149</sup> residue appears only in StoPIMT (Fig. 2). The two monomers forming a disulfide-bridged dimer are related by a noncrystallographic 2-fold axis of symmetry. Three of these disulfide-bridged dimers assemble into a hexamer having crystallographic 3-fold symmetry. Therefore, the StoPIMT hexamer is a trimer of disulfide-bridged dimers with a point group symmetry 32, and two independent hexamers can be generated from the four molecules in the asymmetric unit (Fig. 1C).

In the hexameric structure, three distinct intermolecular interfaces are observed. One is the “major contact,” which corresponds to the interface between two disulfide-linked monomers (red arrows in Fig. 1D). The major contact contains three hydrogen bonds (Thr<sup>149</sup> O<sub>y</sub>–Gly<sup>201</sup> O, Glu<sup>153</sup> O<sub>e</sub>–Glu<sup>153</sup> O<sub>e</sub>, and Val<sup>167</sup> N<sub>e</sub>–Asp<sup>204</sup> O<sub>s</sub>) and one salt bridge (Lys<sup>150</sup> N<sub>e</sub>–Glu<sup>153</sup> O<sub>e</sub>), and the area of interaction is 947 Å<sup>2</sup> (Fig. 1D). Another interface is the “minor contact,” a smaller intermolecular interface between adjacent methyltransferase fold domains, which are related by crystallographic 3-fold symmetry (blue arrows in Fig. 1D). The minor contact has an area of 749 Å<sup>2</sup> and contains only one hydrogen bond (Asn<sup>22</sup> N<sub>O</sub>–His<sup>177</sup> O<sub>e</sub>). The third contact is formed among three identical C-terminal α-helices (α<sub>9</sub>) related by crystallographic 3-fold symmetry.
Fig. 1. Crystal structure of StoPIMT and the hexameric structure in solution. A, stereo ribbon diagram of a monomer of StoPIMT, which consists of an AdoMet-dependent methyltransferase fold domain and a C-terminal α-helical domain. The ribbon model is colored according to the sequence by a rainbow color ramp going from blue at the N terminus to red at the C terminus. Bound AdoHcy is also shown as a ball-and-stick model. B, topology diagrams of a monomer of StoPIMT (top) and typical AdoMet-dependent methyltransferase fold domain of rRNA methyltrans-
Three α-helices form a distinctive coiled-coil assembly and form a hydrophobic core composed of Ile209, Val213, and Leu216, Ile220, and Ile223. The coiled-coil structure seems to be additionally stabilized by one salt bridge between Arg212 and Glu217. This indicates that two monomers of the deletion mutant protein (data not shown), indicating that the hexamer of the truncated mutant is unstable. These results reveal that Tyr203 makes a significant contribution to hexamerization. Additional deletions from StoPIMT-d202 resulted in proteins (data not shown), indicating that the hexamer of the truncated mutant is unstable. These results reveal that Tyr203 makes a significant contribution to hexamerization. Additional deletions from StoPIMT-d202 in proteins (StoPIMT-d199 and StoPIMT-d197) having only monomers and dimers, without hexamers. The addition of a slight molar excess of 2-mercaptoethanol to the fractions corresponding to dimers of StoPIMT-d202, StoPIMT-d199, and StoPIMT-d197 resulted in dissociation of the dimeric structures into monomers (data not shown). This indicates that two monomers of the deletion mutant proteins associate with each other by a disulfide bond without appropriate contact between the major contact regions. Consideration of four molecules (chains A, B, C, and D) in the asymmetric unit (dashed box) and two adjacent hexamers generated by crystallographic symmetry. The hexamer belongs to point group 32 (crystallographic 3-fold + pseudo-2-fold). The asterisks represent the positions of Tyr203 that significantly contribute to hexamerization. The intermolecular disulfide linkage between Cys149 residues and the positions of the major and minor contacts are also shown. A stereo diagram of disulfide-bridged dimer (chains A and B) is shown in Fig. 7A. D, ribbon diagram of hexameric structure of StoPIMT; the 3-fold axis of symmetry is perpendicular to the plane of the paper. The major and minor contact regions are indicated by red and blue triangles, respectively. E, results of size exclusion chromatography of StoPIMT on HiLoad Superdex200. The elution points of standard marker proteins are shown along the top.
ering that the deleted residues from 198 to 205 and the cysteine residue that forms the intermolecular disulfide link are located in the interface of the major contact region, deletion of Tyr203 apparently affects not only the major but also the minor contact interfaces.

**Comparison of Thermostability and Tolerance to GdnHCl of Wild-type and Mutant StoPIMTs**—The thermostability of each protein was measured by differential scanning calorimetry (DSC). Fig. 5A shows DSC results for wild-type StoPIMT and its deletion mutants that have hexameric structures, and Fig. 5B shows the results for deletion mutants that form only monomeric and dimeric structures. Table III summarizes the denaturation enthalpy values and peak temperatures of the profiles.

All of the samples showed irreversible transition and precipitated after the DSC run, so we analyzed the profile mainly in terms of the peak temperatures. The thermogram of wild-type StoPIMT shows a peak at 98.7 °C with a slight shoulder, and those of StoPIMT-d205 and StoPIMT-d204 show peaks at ~101 °C without a shoulder. The peak for StoPIMT-d203 occurs at a lower temperature with a shoulder, and the hexameric fraction of StoPIMT-d202 shows three peaks; in contrast,
TABLE III
Denaturation enthalpy values and peak temperatures of the profiles of StoPIMT wild-type and deletion mutants

| Protein concentration  | $\Delta H$ (kJ/mol monomer) | Peak temperature  | Other peaks |
|------------------------|-----------------------------|-------------------|-------------|
| StoPIMT-WT             | 2.01                        | 655               | 98.7        |
| StoPIMT-d205           | 1.70                        | 555               | 101.0       |
| StoPIMT-d207           | 1.63                        | 585               | 101.7       |
| StoPIMT-d203           | 1.53                        | 561               | 95.0        |
| StoPIMT-d202 hexamer^b | 1.58                        | 839               | 83.9        |
| StoPIMT-d202 monomer^b | 1.56                        | 602               | 85.0        |
| StoPIMT-d199 dimer     | 1.74                        | 434               | 84.8        |
| StoPIMT-d199 monomer   | 1.73                        | 459               | 86.6        |
| StoPIMT-d197 dimer     | 1.36                        | 446               | 86.9        |
| StoPIMT-d197 monomer   | 1.36                        | 424               | 85.6        |
| StoPIMT-WT-mutant^b    | 1.84                        | 643               | 97.6        |
| StoPIMT-d205-mutant^b  | 1.68                        | 627               | 95.9        |

^a The main peak, of which heat capacity change is largest among peaks.
^b The main peak has a shoulder.

Fig. 5. Differential scanning calorimetry of StoPIMT and its deletion mutants. A, heat capacity curves for StoPIMT and its deletion mutants that form hexamers: StoPIMT (red), StoPIMT-d205 (blue), StoPIMT-d204 (green), StoPIMT-d203 (yellow), and the hexameric fraction of StoPIMT-d202 (brown). B, heat capacity curves for StoPIMT and monomeric fractions of its deletion mutants: StoPIMT (red), monomeric fraction of StoPIMT-d202 (blue), StoPIMT-d199 (magenta), and StoPIMT-d197 (green). The hexameric fraction of StoPIMT-d202 is also shown for comparison (dashed brown line).
the monomer fraction of StoPIMT-d202 showed two peaks at
−85 and 90 °C. The monomeric fraction of StoPIMT-d199 and
StoPIMT-d197 showed one peak at −85 °C.

To evaluate the stability of StoPIMT and its mutants to a
chemical denaturant, the effects of GdnHCl on their denatur-
ation were investigated by CD spectroscopy. Fig. 6 shows the
CD intensities at 222 nm for each protein at various GdnHCl
concentrations. The transition midpoints for StoPIMT,
StoPIMT-d205, -d204, -d203, and -d202, were 4.2, 4.5, 3.4, 3.4,
and 3.2 M GdnHCl, respectively. The monomer and dimer frac-
tions of StoPIMT-d202, -d199, and -d197 showed transition
midpoints of −3.0 M GdnHCl.

**Effects of Mutation of Two Residues in the Dimeric Interface on the Thermal/Chemico- stability of StoPIMT**—Thus far, our
results show that the hexamerization of StoPIMT seems to
generally correlate with its thermostability and tolerance to
GdnHCl and that some residues in the major contact region
(i.e. Tyr203-Asp204-Asp205) make critical contributions to the
protein’s hexamerization and thermostability. The crystal
structure of StoPIMT shows that two hydrogen bonds,
Thr146-Glu201 and Asp204-Val167, and one disulfide bond,
Cys149-Cys149, are located in the major contact region (Fig. 7A).
Fig. 7D). The midpoints of the major contact region are critical for hexamerization and hyperthermostability and also suggest that the three residues,
Tyr203-Asp204-Asp205, are critical for hexamerization and
hyperthermostability. As proposed from studies of the effects of
mutations on several types of interactions, including receptor-

**DISCUSSION**

Oligomerization has been suggested to make a critical con-
tribution to the stability of proteins (10, 20–25), and recent
structural studies have demonstrated that many archaeon pro-
teins have homo-oligomeric structures. Some reports have
shown correlation between oligomerization and the hyperther-
mostability of archaeon proteins (20, 21). Oligomerization is
usually through interfacial interactions (34–36), in which sub-
units cooperatively interact with each other in several ways
e.g. domain-swapping and coiled-coil association) (49, 50).
The crystal structure of StoPIMT includes three areas of intermolec-
ular contact (i.e. major, minor, and C-terminal α-helical
coiled-coil contact regions); therefore, the hexameric structure of
StoPIMT has interfacial interactions and coiled-coil associ-
ations. To address how oligomerization of archaeon proteins
contributes to their hyperthermostability, here we focused on
StoPIMT.

**Hexamerization of StoPIMT: Correlation with Thermostabil-
ity and Existence of Hot Spots**—Analyses of the oligomerization
states of wild-type and mutant StoPIMT proteins indicates that
the normal hexameric structure is retained even after deletion of the C-terminal α-helical domain; dissociation of
hexamers into monomers and dimers occurred only after re-
moval of the next three residues (Tyr203-Asp204-Asp205). SEC
analyses indicated that the presence of Tyr203 is critical for
oligomerization of the protein, and DSC measurements indi-
cated the critical contribution of Asp204 to the thermostability
of the protein. Deletion of Asp205 resulted in decreased toler-
ance to GdnHCl. In the crystal structure of StoPIMT, the loop
region including these residues is located in the major contact
region (Figs. 1D and 7A). Replacement of some residues in the
minor contact region with Ala affected neither the thermosta-
B and 7C. DSC measurements also showed two
peaks for the double mutants; one peak is identical to that for
the unstable hexameric structure of StoPIMT-d202 and has a
maximum at 90.7 °C, and the other is from the same as that for
hexamers of wild-type and mutants that have maximums at
−98 °C (Fig. 7D).
ligand interactions (51, 52), antigen-antibody interactions (53, 54), and homodimer formation (55, 56), we propose that these residues also be defined as hot spot residues (57) for hexamerization and/or hyperthermostability.

Formation of oligomeric structures was shown to be correlated with enhanced stability of StoPIMT. The electrostatic potential on the surface of the minor contact region of the StoPIMT monomer is relatively neutral, although that of the major contact region is relatively negatively charged (Fig. 8). Accordingly, association between the neutral surfaces of the minor contacts of two monomers leads to a decrease in neutral surface area and exposure of relatively more charged surfaces of the protein to the solvent. It has been proposed that an increased number of polar residues at the molecular surface enhances protein thermostability (3–10). Enhanced thermostability in StoPIMT may thus be partly due to the decrease in neutral surface area and the consequent increase in charged surface area that it can achieve by hexamerization. We should note that the minor contact region of one monomer cannot interact with its counterpart on another monomer without appropriate contact between the major contact regions, especially including Tyr203-Asp204-Asp205.

The stabilities of StoPIMT and StoPIMT-d205 in the presence of GdnHCl were decreased by replacing Thr146 and Asp204 with Ala; they became almost as easily denatured as the deletion mutant StoPIMT-d202. StoPIMT-WT-mutant and StoPIMT-d205-mutant had a peak at 90°C in the DSC analyses, which was not observed in the wild-type and mutant proteins having stable hexameric structures, suggesting that deletion of some interactions in the major contact caused some structural changes. The significant decreases in the tolerance to GdnHCl of StoPIMT-WT-mutant and StoPIMT-d205-mutant might be correlated with the appearance of this peak in the DSC analyses of the mutants. The two replaced residues are involved in intermolecular contacts in the major contact region (Fig. 7A). The significant decrease in thermostability and tolerance to GdnHCl resulting from these mutations indicates the critical contribution of the side chains of Thr146 and Asp204 to stability via hydrogen bond formation. These mutants, however, do have hexameric structures, suggesting that the thermostability and tolerance to GdnHCl of StoPIMT may be triggered by favorable interactions in the major contact region, including hydrogen bond formation by these two residues. We should note that Asp204 is located in the C-terminal loop, which was shown to be essential for hexamerization. Therefore, we could conclude that the protein is hexamerized via the C-
terminal loop including Tyr<sup>203</sup>-Asp<sup>204</sup>-Asp<sup>205</sup> and that thermostability can be achieved by rearrangement of the contacts of Thr<sup>146</sup> and Asp<sup>204</sup> in the major contact region. These results again suggest the existence of hot spot residues for the formation of hexamers and acquisition of thermostability. We should also note that cooperative association of monomers into a hexamer via the hot spot residues is important for the thermostable architecture of the protein.

**Contribution of C-terminal Coiled-coil Contact Region to Hexamerization and Thermostability of StoPIMT**—The thermostability and tolerance to GdnHCl of StoPIMT-d205 and StoPIMT-d204 were greater than that of StoPIMT, despite deletion of the α<sub>8</sub>-helix, which participates in the trimerized coiled-coil structure. In the crystal structure, the asymmetric unit contains four molecules of StoPIMT. Their superimposition on the AdoMet-dependent methyltransferase fold domain of each protein revealed that the C-terminal α-helical domains, especially the N and C termini of α<sub>8</sub>, did not overlap well on each other. The C-terminal α-helical domain of StoPIMT is flexible, and therefore packing within a hexamer via the coiled-coil structure is relatively loose in comparison with the major and minor contact regions. Deletion of this flexible domain in StoPIMT leads to some enhancement of hyperthermostability; for example, Russell et al. (11) reported that shortening a loop region is one of the factors that increase thermostability in citrate synthase from thermophiles. Thus, one may conclude that oligomerization of proteins via association of coiled-coil regions does not enhance their stability but may lead to slight destabilization.

**Structural Comparison of StoPIMT with PfuPIMT and Human PIMT and Its Relation to Thermostability**—The crystal structure of PfuPIMT has been reported (29), revealing that PfuPIMT has a monomeric structure. Although the root mean square difference of StoPIMT superimposed on PfuPIMT is only 1.23 Å, and their amino acid sequence homology is greater than 35%, PfuPIMT has higher thermostability than StoPIMT. The crystal structure of PfuPIMT may be characterized as including intramolecular hydrogen bonds, ion pairs on the protein surface, and less hydrophobic surface with many charged residues than StoPIMT. The numbers of intramolecular hydrogen bonds whose lengths were within 3.0 Å of ion pairs whose lengths were within 3.3 Å when StoPIMT was superposed on homologous sequences in PfuPIMT and human PIMT were thus calculated and compared. There were 120 hydrogen bonds in StoPIMT, but 147 and 145 in PfuPIMT and human PIMT, respectively. Nine and five ion pairs were observed in (monomeric) PfuPIMT and human PIMT, respectively, but the numbers of ion pairs in four StoPIMT monomers within an asymmetric unit in the crystal structure were four (three molecules) and six (one molecule). These results indicate that thermostability of StoPIMT does not originate from increases in numbers of intramolecular hydrogen bonds and ion pairs. We can therefore consider that oligomerization may be an alternative means for the development of thermal stability in StoPIMT. It is interesting to note that different mechanisms make PIMT hyperthermostable in the two archaeons.

**Conclusions**—X-ray crystallography of StoPIMT has revealed its hexameric structure. The hexameric associations resulted from three interfacial contacts: major, minor, and coiled-coil contact regions. Although the C-terminal α-helical domain promotes multimerization of StoPIMT by interacting to form coiled coils, this domain is not needed for hexamerization of StoPIMT and does not contribute importantly to increasing its thermostability. Mutation in and/or deletions that include the major contact regions affect the oligomerization and thermostability of StoPIMT, suggesting that local structures within the major contact region contribute importantly to oligomerization and to increasing the protein's stability via hexamerization. The existence of interfacial hot spot residues for promoting thermostability via oligomerization and cooperative hexamerization via interactions of two hot spot residues are suggested.

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**REFERENCES**

1. Chakravarty, S., and Varadarajan, R. (2002) *Biochemistry* **41**, 8152–8161
2. Chan, M. K., Mukund, S., Kletzin, A., Adams, M. W., and Rees, D. C. (1995) *Science* **267**, 1463–1469
3. Hennig, M., Sterner, R., Kirschner, K., and Jansonius, J. N. (1997) *Biochemistry* **36**, 6009–6016
4. Hennig, M., Darimont, B., Sterner, R., Kirschner, K., and Jansonius, J. N. (1995) *Structure* **3**, 1295–1306
5. Sanchez-Ruiz, J. M., and Makhatadze, G. I. (2001) *Trends Biotechnol.* **19**, 132–135
6. Lolasdez, V. V., Ibarra-Molero, B., Sanchez-Ruiz, J. M., and Makhatadze, G. I. (1999) *Biochemistry* **38**, 16419–16423
7. Tsuchiya, T. H., Oki, H., Tsukihara, T., Ogashara, K., Yutani, K., Ogata, K., Izu, Y., Tsunawasa, S., and Kato, I. (1998) *J. Mol. Biol.* **284**, 101–124
8. Cambillau, C., and Claverie, J. M. (2000) *J. Biol. Chem.* **275**, 32383–32386
9. Konradt, I., Steipe, B., Huber, R., Tomasch, A., and Jaenicke, R. (1995) *J. Mol. Biol.* **246**, 511–521
10. Jaenicke, R., and Bohm, G. (1998) *Curr. Opin. Struct. Biol.* **8**, 738–748
11. Russell, R. J., Hough, D. W., Danson, M. J., and Taylor, G. L. (1994) *Structure* **2**, 1157–1167
12. Britton, K. L., Baker, P. J., Borges, K. M., Engel, P. C., Pasquo, A., Rice, D. W., Robb, F. T., Scandurra, R., Stillman, T. J., and Yip, K. S. (1995) *Eur.
Hot Spots for Oligomerization and Thermostability of Protein

J. Biochem. 229, 688–695

13. Hatanaka, H., Tanimura, B., Katoh, S., and Inagaki, F. (1997) J. Mol. Biol. 268, 922–933

14. Spassov, V. Z., Karashkoff, A. D., and Ladenstein, R. (1995) Protein Sci. 4, 1516–1527

15. Schumann, J., Bohm, G., Schumacher, G., Rudolph, R., and Jaenicke, R. (1993) Protein Sci. 2, 1612–1620

16. Russell, R. J., Ferguson, J. M., Hough, D. W., Danzon, M. J., and Taylor, G. L. (1997) Biochemistry 36, 9883–9994

17. Tanner, J. J., Hecht, R. M., and Krause, K. L. (1996) Biochemistry 35, 2597–2609

18. Vogt, G., and Argos, P. (1997) Fold Des. 2, S40–S46

19. Li, T., Sun, F., Ji, X., Peng, Y., and Rao, Z. (2003) J. Mol. Biol. 325, 1031–1037

20. Ogasahara, K., Khochanakhril, N. N., Nakamura, M., Yoshimoto, T., and Yutani, K. (2001) Eur. J. Biochem. 268, 3233–3242

21. Thoma, K., Hennig, M., Sterner, R., and Kirschen, K. (2000) Struct. Fold Des. 8, 265–270

22. Villeret, V., Clantin, B., Tricot, C., Legrain, C., Roovers, M., Stalon, V., Glansdorff, N., and Van Beeumen, J. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 2801–2806

23. Sterner, R., Kleemann, G. R., Szadkowski, H., Lustig, A., Hennig, M., and Kirschen, K. (1996) Protein Sci. 5, 2000–2008

24. Ogasahara, K., Ishida, M., and Yutani, K. (2003) J. Biol. Chem. 278, 8922–8928

25. Walden, H., Bell, G. S., Russell, R. J., Siebers, B., Hensel, R., and Taylor, G. L. (2001) J. Mol. Biol. 306, 745–757

26. Geiger, T., and Clarke, S. (1987) J. Biol. Chem. 262, 785–794

27. Thapar, N., Griffith, S. C., Yeates, T. O., and Clarke, S. (2002) J. Biol. Chem. 277, 1058–1065

28. Skinner, M. M., Puvathingal, M. R., Bajer, M., and Friedman, A. M. (2000) Struct. Fold Des. 8, 1189–1201

29. Griffith, S. C., Sawaya, M. R., Boutz, D. R., Thapar, N., Kats, J. E., Clarke, S., and Yeates, T. O. (2001) J. Mol. Biol. 313, 1103–1116

30. Smith, C. D., Carson, M., Friedman, A. M., Skinner, M. M., Delucia, L., Chantarat, L., Weise, L., Shirasawa, T., and Chattopadhyay, D. (2002) Protein Sci. 11, 625–635

31. Rytersgaard, C., Griffith, S. C., Sawaya, M. R., MacLaren, D. C., Clarke, S., and Yeates, T. O. (2002) J. Biol. Chem. 277, 10642–10646

32. Bennett, E. J., Bjerregaard, J., Knapp, J. E., Chavous, D. A., Friedman, A. M., Royer, W. E., Jr., and O'Connor, C. M. (2003) Biochemistry 42, 12844–12853

33. Cheng, X., and Roberts, R. J. (2001) Nucleic Acids Res. 29, 3784–3795

34. Jaenicke, R., and Lilie, H. (2000) Adv. Protein Chem. 53, 329–401

35. Schlanegger, M. P., Bennett, M. J., and Eisenberg, D. (1997) Adv. Protein Chem. 50, 61–122

36. Rousseau, F., Schymkowitz, J. W., and Itzhaki, L. S. (2003) Structure (Camb.) 11, 243–251

37. Tsutomu, K., Shinoki, K., Kondo, H., Uchikawa, M., Juji, T., and Kumagai, I. (1998) J. Immunol. Methods 219, 119–129

38. Tanaka, Y., Tsutomu, K., Nakamura, T., Yasukawa, Y., Sakai, N., Yao, M., Tanaka, I., and Kumagai, I. (2004) FEBS Lett. 556, 167–174

39. Studier, F. W., and Moffatt, B. A. (1986) J. Mol. Biol. 189, 113–130

40. Budisa, N., Steipe, B., Demange, P., Eckerskorn, C., Kellermann, J., and Huber, R. (1995) Eur. J. Biochem. 230, 788–796

41. Leslie, A. G. W. (1992) Joint CCP4 and ESF-EMBC Newsletter on Protein Crystallography, p. 26, SERC Daresbury Laboratory, Warrington, UK

42. Collaborative Computational Project, N. (1994) Acta Crystallogr. Sect. D Biol. Crystallogr. 50, 760–763

43. Terwilliger, T. C., and Berendzen, J. (1999) Acta Crystallogr. Sect. D Biol. Crystallogr. 55, 849–861

44. Terwilliger, T. C., and Berendzen, J. (1999) Acta Crystallogr. Sect. D Biol. Crystallogr. 55, 1872–1877

45. Terwilliger, T. C. (2000) Acta Crystallogr. Sect. D Biol. Crystallogr. 56, 955–972

46. Jones, T. A., Zou, J. Y., Cowan, S. W., and Kjeldgaard, H. (1991) Acta Crystallogr. Sect. A 47, 110–119

47. Brugger, A. T., Adams, P. D., Clore, G. M., Delano, W. L., Gros, P., Grosse-Kunstleve, R. W., Jiang, J. S., Kuszewski, J., Nilges, M., Pannu, N. S., Read, R. J., Rice, I. M., Simonson, T., and Warren, G. L. (1998) Acta Crystallogr. Sect. D Biol. Crystallogr. 54, 965–972

48. Laskowski, R. A., MacArthur, M. W., Moss, D. S., and Thornton, J. M. (1993) J. Appl. Crystallogr. 26, 283–291

49. Burkhard, P., Stetefeld, J., and Strelkov, S. V. (2001) Trends Cell Biol. 11, 82–88

50. Arndt, K. M., Muller, K. M., and Pluckthun, A. (2001) J. Mol. Biol. 312, 221–228

51. Clackson, T., and Wells, J. A. (1995) Science 267, 383–386

52. Pohler, J., Ruissman, L. C., and Schreiber, G. (2000) J. Biol. Chem. 275, 40425–40433

53. Tsutomu, K., Ogasahara, K., Ueda, Y., Watanabe, K., Yutani, K., and Kumagai, I. (1995) J. Biol. Chem. 270, 18551–18557

54. L. Y., Urrutia, M., Smith-Gill, S. J., and Mariuzza, R. A. (2003) Biochemistry 42, 11–22

55. Sengchanthalangsy, L. L., Datta, S., Huang, D. B., Anderson, E., Braswell, E. H., and Ghosh, G. (1999) J. Mol. Biol. 289, 1029–1040

56. Hart, D. J., Speight, R. E., Sutherland, J. D., and Blackburn, J. M. (2001) J. Mol. Biol. 310, 563–575

57. Bogan, A. A., and Thorn, K. S. (1998) J. Mol. Biol. 280, 1–9

58. Bussiere, D. E., Muchmore, S. W., Dealwis, C. G., Schluckebier, G., Nienaber, V. L., Edalji, R. P., Walter, K. A., Ladror, U. S., Holzman, T. F., and Abad-Zapatero, C. (1998) Biochemistry 37, 7103–7112

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