Structural Characterization of an ACP from *Thermotoga maritima*: Insights into Hyperthermal Adaptation

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Abstract: *Thermotoga maritima*, a deep-branching hyperthermophilic bacterium, expresses an extraordinarily stable *Thermotoga maritima* acyl carrier protein (*Tm*-ACP) that functions as a carrier in the fatty acid synthesis system at near-boiling aqueous environments. Here, to understand the hyperthermal adaptation of *Tm*-ACP, we investigated the structure and dynamics of *Tm*-ACP by nuclear magnetic resonance (NMR) spectroscopy. The melting temperature of *Tm*-ACP (101.4 °C) far exceeds that of other ACPs, owing to extensive ionic interactions and tight hydrophobic packing. The D59 residue, which replaces Pro/Ser of other ACPs, mediates ionic clustering between helices III and IV. This creates a wide pocket entrance to facilitate the accommodation of long acyl chains required for hyperthermal adaptation of the *T. maritima* cell membrane. *Tm*-ACP is revealed to be the first ACP that harbor an amide proton hyperprotected against hydrogen/deuterium exchange for I15. The hydrophobic interactions mediated by I15 appear to be the key driving forces of the global folding process of *Tm*-ACP. Our findings provide insights into the structural basis of the hyperthermal adaptation of ACP, which might have allowed *T. maritima* to survive in hot ancient oceans.

Keywords: Acyl carrier protein; NMR spectroscopy; structure; thermostability; *Thermotoga maritima*

1. Introduction

Acyl carrier proteins (ACPs) are small (~9 kDa) acidic proteins that are essential for numerous biochemical pathways, including the biosynthesis of fatty acids, polyketides, lipopolysaccharides, lipoteichoic acids, rhizobial nodulation signaling factors, and pro-hemolysin toxins [1–6]. Fatty acid synthesis (FAS) is an essential process that produces fatty acids, which are important energy sources for cells and also serve as the building blocks of cell membranes and intracellular signaling substances [7]. As essential components in type II FAS systems [1,8], ACPs shuttle acyl intermediates in their hydrophobic pocket to facilitate interactions with various enzyme partners [9,10]. ACPs share highly conserved structures, including Asp-Ser-Leu (DSL) motifs at the N-terminus of helix II. A phosphopantetheine group is attached to the Ser residue (purple box in Figure 1a) by a phosphodiester linkage, and the free thiol group at the other end of the phosphopantetheine group can form a thioester bond with acyl groups [11–14]. The key structural features of various type II ACPs have been reported [15–23]. ACPs consist of four helical bundles connected by three loop regions, forming hydrophobic cavities to accommodate the growing acyl chains. ACPs are believed to be dynamic proteins, and their flexibilities are essential for their functions [10,24–28].
Acidic packed hydrophobic interactions, which help to maintain proper folding at high temperatures. Enriched in saturated fatty acids with long chain lengths, which help maintain their liquid crystalline states at high temperatures. A bacterium known to grow at temperatures up to 90 °C is a valuable target since Thermotoga maritima is known for its functional flexibility in such harsh environments. In this respect, Thermophilic proteins often contain extra salt bridges or tightly packed hydrophobic interactions, which help to maintain proper folding at high temperatures. Proteins must maintain not only the correct structure but also the appropriate dynamic motion to accomplish their functions. Thermophilic proteins often contain extra salt bridges or tightly packed hydrophobic interactions, which help to maintain proper folding at high temperatures.

Protein structures have evolved to facilitate the adaption of the expressing organism to various environments. Proteins must maintain not only the correct structure but also the appropriate dynamic motion to accomplish their functions. Thermophilic proteins often contain extra salt bridges or tightly packed hydrophobic interactions, which help to maintain proper folding at high temperatures [29,30]. It is important to understand the mechanisms through which proteins balance structural stability and functional flexibility in such harsh environments. In this respect, Thermotoga maritima ACP (Tm-ACP) is a valuable target since T. maritima resides in extremely hot hydrothermal vents and is the only bacterium known to grow at temperatures up to 90 °C [31]. Its thermophilic cell membrane is highly enriched in saturated fatty acids with long chain lengths, which help maintain their liquid crystalline states at high temperatures [32,33]. Therefore, identification of the physicochemical properties of the

**Table 1.** Comparison of isoelectric points and the number of charged residues of bacterial ACPs. (https://web.expasy.org/protparam)

**Figure 1.** Sequence alignment of bacterial ACPs and the thermostability of Thermotoga maritima acyl carrier protein (Tm-ACP). (a) Sequence alignment of Tm-ACP with other bacterial ACPs, including hyperthermophilic Pt-ACP, thermophilic Ta-ACP, and mesophilic Ef-ACP, Bm-ACP, Ec-ACP, and Vh-ACP. 100% conserved residues are highlighted as black color, above 80% conservations are boxed by black lines. Mutated sites in this study are indicated by asterisks. All ACPs have conserved Ser residues that are linked to phosphopantetheine linkers (purple box). Unlike mesophilic proteins, hyperthermophilic ACPs have additional basic residues and nonconserved Asp residues, which are indicated by blue and red boxes, respectively. Tm-ACP has three Phe residues (orange boxes): one is highly conserved in all ACPs (F54) and two are rare (F8 and F50). I15 in hyperthermophilic ACPs have additional basic residues that are

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hyperthermophilic ACP could provide important clues for understanding the functioning of the FAS system at extremely high temperatures.

In order to gain insights into the thermophilic adaptation of the deep-branching bacterium T. maritima for surviving in hot aqueous environments, we investigated the structural factors contributing to the hyperthermostability of Tm-ACP. To this end, we investigated its melting temperature (T_m) by differential scanning calorimetry (DSC) and its structural features by nuclear magnetic resonance (NMR) spectroscopy. Moreover, the thermodynamic parameters related to the folding of Tm-ACP were analyzed by NMR and circular dichroism (CD) experiments. These data will offer important clues for studying evolutionary strategies of the hyperthermophilic adaptation of T. maritima at the molecular level.

2. Results

2.1. Thermostability of Tm-ACP

Hyperthermophilic Tm-ACP shows high sequence similarities with other bacterial ACPs, ranging from 65.4% to 78.8% (Figure 1a). Tm-ACP has the conserved DSL motif (purple box in Figure 1a), where the Ser residue becomes connected to the phosphopantetheine linker. Despite this high sequence similarity, Tm-ACP also shows some unique sequence characteristics. Notably, although all bacterial ACPs have about 20 acidic residues, three thermophilic ACPs have double the number of positively charged residues compared to that found in mesophilic ACPs (Table 1). The nonconserved basic residues (blue boxes in Figure 1a) are located in various regions of Tm-ACP. The hyperthermophilic Tm-ACP and Pt-ACP both contain an Asp residue in the α2α3 loop which substitutes for the Pro or Ser of most mesophilic ACPs (red box in Figure 1a). Tm-ACP also has three Phe residues (orange boxes in Figure 1a), including the highly conserved F54 residue and two nonconserved residues, F8 and F50, resulting in increase of the structural stability of Tm-ACP.

The CD data demonstrated that Tm-ACP starts to denature at approximately 100 °C (Figure 1b). After cooling down the denatured sample to 25 °C, the α-helical structure was completely recovered, implying that the folding reaction of Tm-ACP is reversible. The specific T_m of Tm-ACP was measured as 101.4 °C by DSC (Figure 1c), which is the highest melting temperature for any ACP reported to date [15,18,34]. The reversibility was further confirmed by NMR spectroscopy, in which the 1H-15N heteronuclear single-quantum coherence (HSQC) spectrum of the Tm-ACP sample heated in boiling water for 15 min was identical to that of the native Tm-ACP (Figure S1a).

| Table 1. Comparison of isoelectric points and the number of charged residues of bacterial ACPs. |
|---------------------------------------------------------------|
| Tm-ACP 1 | Pt-ACP 2 | Ta-ACP 3 | Ef-ACP 4 | Bm-ACP 5 | Ec-ACP 6 | Vh-ACP 7 |
|--------------------------|--------------------------|--------------------------|--------------------------|--------------------------|--------------------------|--------------------------|
| pI 8 | 4.13 | 4.28 | 4.29 | 3.87 | 3.97 | 3.98 | 3.79 |
| Acidic (Glu/Asp) | 21 (10/11) | 19 (8/11) | 21 (14/7) | 21 (11/10) | 20 (9/11) | 20 (14/6) | 22 (14/8) |
| Basic (Arg/Lys) | 9 (1/8) | 10 (1/9) | 10 (2/8) | 5 (1/4) | 6 (1/5) | 5 (1/4) | 6 (1/5) |

1 Thermotoga maritima ACP; 2 Pseudothermotoga thermarum ACP; 3 Thermus aquaticus ACP; 4 Enterococcus faecalis ACP; 5 Brucella melitensis ACP; 6 Escherichia coli ACP; 7 Vibrio harveyi ACP; 8 The isoelectric points (pI) of bacterial ACPs were calculated by ProtParam tool (https://web.expasy.org/protparam) [35].

2.2. Tertiary Structure of Tm-ACP

To function as a carrier for acyl groups, an apo Tm-ACP should be converted to the holo form, where the phosphopantetheine linker is covalently connected to the side chain of S40 in the conserved DSL motif [12–14]. Both tertiary structures of the holo and apo forms of Tm-ACP were determined by NMR spectroscopy and X-ray crystallography, respectively (see associated statistics in Table 2; Table 3). In the final calculation of the solution structure of holo Tm-ACP, the well-superimposed 20 lowest-energy models with small root-mean-square deviations (RMSDs; 0.3 and 0.7 Å) were obtained for all backbone and heavy atoms, respectively (Figure 2a). The overall completeness of assignment was 93% and the quality factor (Q) of residual dipolar coupling (RDC) data was calculated as 39.52%
using \( Q = \frac{\text{rms}(\text{RDC}_{\text{measured}} - \text{RDC}_{\text{calculated}})}{\text{rms}(\text{RDC}_{\text{measured}})} \) \cite{36}. \( Tm\)-ACP consists of four \( \alpha \)-helices (helix I (4–19), helix II (40–54), helix III (60–65), helix IV (69–80)) connected by a long \( \alpha_1\alpha_2 \) loop and two shorter loops. To accommodate the acyl chains, the hydrophobic cavities of ACPs can be expanded by protruding helix III outward \cite{1,28,37}. Compared to the structure of holo \( Ec\)-ACP \cite{38}, the helix III of \( Tm\)-ACP was found to be protruded outward in both the apo and holo forms (Figure 2b and Figure S2). Hyperthermophilic \( Tm\)-ACP has nonconserved Glu and Lys residues (D59 and K79), which participate in an ionic cluster between helices III and IV (Figure 2c), forcing the outward protrusion of helix III.

The Table 2. Statistics of solution structure of holo \( Tm\)-ACP. The 20 lowest-energy structures were determined by NMR spectroscopy.

| Restraints \(^1\) | \( \text{Total} \) |
|------------------|----------------|
| Conformationally restricting distance constraints | 1108 |
| Short Range \((i - j) \leq 1\) | 301 |
| Medium Range \((1 < (i - j) \leq 5)\) | 266 |
| Long Range \(((i - j) > 5)\) | 196 |
| Dihedral angle constraints | 77 |
| Phi | 77 |
| Psi | 77 |
| Hydrogen-bond constraints | 114 |
| Residual dipolar coupling (RDC) constraints | 77 |
| Xplor-NIH pseudo-potential energy (kJ/mol) \(^2\) | 3251 |

| Average Rmsd to the Mean Coordinates (Å) \(^3\) |
|----------------------------------|
| Backbone atoms (all / ordered residues \(^4\)) | 0.3/0.2 |
| Heavy atoms (all / ordered residues \(^4\)) | 0.7/0.6 |

| Ramachandran Plot Summary from PROCHECK (%) \(^3\) |
|----------------------------------|
| Most favored regions | 96.4 |
| Allowed regions | 3.6 |
| Disallowed regions | 0.0 |

| Average Number of Violations Per Conformer \(^2\) |
|----------------------------------|
| Distance constraint violations \((> 0.2 \text{ Å})\) | 0 |
| Angle constraint violations \((> 10^\circ)\) | 0 |

\(^1\) The solution structure of holo \( Tm\)-ACP was calculated using Xplor-NIH-based calculation in PONDEROSA-C/S \cite{39}. \(^2\) Xplor-NIH pseudo-potential energy and all violations of the 20 best structures were analyzed using PONDEROSA-Analyzer \cite{40}. \(^3\) The final 20 lowest-energy structures were analyzed using PSVS (Protein Structure Validation Software) \cite{41}. \(^4\) Ordered residues: S3-L80.

The \( Tm\)-ACP structure has extensive electrostatic interactions in four different regions (Figure 2c). In region I (yellow), a nonconserved R4 residue forms ionic interactions with E28 and E77. In addition, E6 makes a salt bridge with K10. In region II (green), two ionic clusters, D22-K12-E23 and E17-K18-D49, further stabilize the C-terminal half of helix I, along with helix II and the long \( \alpha_1\alpha_2 \) loop. In region III (purple), a salt bridge between nonconserved residues K31 and D34 stabilizes a helical turn (L32–L36) located in the middle of the long \( \alpha_1\alpha_2 \) loop, thereby increasing the rigidity of this loop. Finally, in region IV (orange), the \( \alpha_2\alpha_3 \) loop and three helices, II, III and IV, are connected by extensive ionic interactions, including E51-K57 and D59-K65-D62-K79. This region is reported as the divalent cation-binding site and usually consists of many Glu residues in mesophilic ACPs \cite{18,42}. The nonconserved K57 residue in hyperthermophilic \( Tm\)-ACP replaces these Glu residues, resulting in stabilization of the short \( \alpha_2\alpha_3 \) loop.
with additional Phe residues, F8 and F50 (Figure 1a). Similar to most ACP structures, F54 of Tm-ACP (yellow), where the backbone atoms of the helices are aligned (RMSD = 1.13 Å), (b) Superimposition of two structures: the solution structure of holo Tm-ACP (blue) with the lowest energy and the crystal structure of apo Tm-ACP (yellow), where the backbone atoms of the helices are aligned (RMSD = 1.13 Å). (c) Electrostatic interactions in Tm-ACP divided into four different regions depicted in yellow, green, purple, and orange, respectively. Residues, which constitute the ionic cluster between helix III and IV (D59-K65-D62-K79), are labeled red. The positively charged nitrogen atoms in the guanidyl group of R4 and amino group of Lys residues form electrostatic interactions with the negatively charged oxygen atoms in the carboxylic groups of Asp and Glu residues. (d) Hydrophobic packing between three helices: I, II, and IV. The hydrophobic side chains of the packing residues are shown in yellow, except for the Phe residues (orange) and I15 (red).

The interior of Tm-ACP is filled with numerous hydrophobic side chains, in which three Phe residues and I15 mediate the tight hydrophobic packing (Figure 2d). Located at the center of the packing, the hydrophobic side chain of I15 contact closely with those of 8 residues, V11 (2.1 Å), L19 (2.1 Å), V26 (3.1 Å), L32 (2.1 Å), L36 (2.4 Å), L46 (2.1 Å), F50 (2.4 Å), and V69 (3.1 Å). The shortest distances of each side chain from that of I15 were measured between the two closest protons, giving the average distance of 2.4 Å. Tm-ACP has a highly conserved aromatic F54 residue at the end of helix II along with additional Phe residues, F8 and F50 (Figure 1a). Similar to most ACP structures, F54 of Tm-ACP forms a hydrophobic triad with I7 and I76 at the top end of the hydrophobic cavity, fastening three
helices: I, II, and IV. Nonconserved F8 at helix I forms hydrophobic contacts to V26 in the long \( \alpha_1 \alpha_2 \) loop, and V69 and V73 in helix IV, further stabilizing the flexible \( \alpha_1 \alpha_2 \) loop. Aromatic rings in F50 and F54 form additional hydrophobic interactions with other residues (I7, V11, I14, I15, L46, I72, I76, and L80) in the hydrophobic cavity. Nuclear Overhauser effects (NOEs) were observed for F50 with V11, I14, and I15, implying strong connections between helices I and II.

### Table 3. Statistics of crystal structure of apo Tm-ACP.

| Data collection |  |
|-----------------|-----------------|
| Space group     | P2\(_1\)2\(_1\)2\(_1\) |
| Unit-cell       | a, b, c (Å)     |
|                 | 23.83, 61.95, 95.72 |
|                 | \( \alpha, \beta, \gamma \) (\( ^\circ \)) |
|                 | 90, 90, 90 |
| Resolution (Å)  | 28.37–2.29 |
| Unique reflections | 49,345 |
| Redundancy      | 7.3 |
| Completeness for range (%) | 98.2 |
| Mean I/\( \sigma(I) \) | 26.65 (at 2.29 Å) |
| R\(_\text{merge} \) (%) | 8.1 |

### Refinement

| No. of reflections (overall) | 6797 |
| No. of reflections (test set) | 671 |
| R-factor \(^3\) | 0.2217(0.2104 at 2.29 Å) |
| R-free \(^4\) | 0.2841(0.2762 at 2.29 Å) |
| RMSZ / RMSD | |
| Bond lengths (Å) | 0.42/0.008 |
| Bond angles (\( ^\circ \)) | 0.63/1.086 |
| Wilson B-factor (Å\(^2\)) | 19.5 |

### Ramachandran Plot Summary from PROCHECK (%)

| Region | Percentage |
|--------|------------|
| Most favored regions | 97.2 |
| Allowed regions | 2.8 |
| Disallowed regions | 0.0 |

### Average B-factors (Å\(^2\))

| Component | B-factor |
|-----------|----------|
| Protein   | 27.3     |
| Water     | 27.9     |
| Ligand    | 40.0     |

\(^1\) \( R_{\text{merge}} = \Sigma hkl \Sigma i (I_i - I_m)^2 / \Sigma hkl \Sigma i I_m^2 \), where \( I_i \) is the \( i \)-th measurement and \( I_m \) is the weighted mean intensity of the reflection. \(^2\) Evaluated by PSVS (Protein Structure Validation Software) \(^4\). \(^3\) \( R \) is calculated as the R-factor for 5% of the data randomly omitted from the refinement. \(^5\) RMSZ is the root-mean-square of all Z scores of the bond lengths (or angles). \(^4\) R-free was calculated as the R-factor for 5% of the data randomly omitted from the refinement. \(^6\) An estimate of the overall B-value of the structure, calculated from the diffraction data. \(^7\) The mean B-value calculated over the modelled atoms by using MOLEMAN2 [43].

### 2.3. Key Residues Contributing to the Thermostability of Tm-ACP

To assess the roles of specific residues on the hyperthermal stability of Tm-ACP, the \( T_m \) of mutant Tm-ACPs were measured using DSC (Table 4). Mutations that replace residues involved in attractive electrostatic interactions by oppositely charged residues reduced the \( T_m \) by 4.2–23.5 \( ^\circ \)C. As R4 connects helix I, helix IV, and the \( \alpha_1 \alpha_2 \) loop, the R4E mutant had much lower thermostability (\( T_m = 86.1 \) \(^\circ \)C) compared to that of the wild-type protein. Replacement of K10 and K18 with Glu residues disrupted favorable electrostatic interactions, consequently reducing the \( T_m \) by 20.0 \(^\circ \)C and 18.3 \(^\circ \)C, respectively. In addition, since K12 stabilizes the \( \alpha_1 \alpha_2 \) loop by forming salt bridges with D22 and E23, the replacement of K12 with Glu caused the most significant decrease in the thermostability of Tm-ACP, resulting in a \( T_m \) of 76.9 \(^\circ \)C. Similarly, additional salt bridges formed by nonconserved
Lys residues, K31 in the $\alpha_1\alpha_2$ loop and K57 in the $\alpha_2\alpha_3$ loop, appear to substantially contribute to the thermostability of $Tm$-ACP by stabilizing each loop. K31E and K57E mutants had reduced $T_m$’s, 90.0 °C and 95.8 °C, respectively. Lastly, the K79E mutation caused the loss of the ionic cluster D59-K65-D62-K79, thereby reducing the thermostability of $Tm$-ACP by 10.6 °C.

| Related Interaction | Phenotype | $T_m$ (°C) |
|---------------------|-----------|------------|
| Electrostatic       | Wild-type | 100.4      |
|                     | R4E       | 86.1       |
|                     | K10E      | 80.4       |
|                     | K12E      | 76.9       |
|                     | K18E      | 82.1       |
|                     | K31E      | 90.0       |
|                     | K57E      | 95.8       |
|                     | K79E      | 89.8       |
| Hydrogen bond       | S16G      | 94.0       |
| Hydrophobic         | F8A       | 94.1       |
|                     | F50A      | 90.7       |
|                     | F54A      | 81.8       |
|                     | V11A      | 91.6       |
|                     | I15A      | 84.6       |
|                     | I72A      | 90.1       |
|                     | V73A      | 89.7       |

As the contribution of the prosthetic group to the thermostability of $Tm$-ACP was found to be negligible, all $T_m$ values of mutants were measured and compared in their apo forms.

Previously, we have reported that a novel ACP from a heat-tolerant mesophile Enterococcus faecalis (Ef-ACP) has a high melting temperature of 78.8 °C [18]. One of the key structural components that contribute to the high thermostability of Ef-ACP was revealed to be a nonconserved hydrogen bond between the side chain of S15 and the backbone of I20 in $\alpha_1\alpha_2$ loop. Similarly, S16 in the hyperthermophilic $Tm$-ACP also forms a hydrogen bond with the backbone of V21, and thus, further stabilizes the $\alpha_1\alpha_2$ loop along with the ionic interactions. As in the mesophilic Ec-ACP [38], the S16G mutant $Tm$-ACP lacking this hydrogen bond, resulting in a reduced $T_m$ of 94.0 °C.

Hydrophobic interactions were also found to be important for the thermostability of $Tm$-ACP. We confirmed that three Phe residues contribute to its high thermostability. The F8A mutation resulted in destabilization of the $\alpha_1\alpha_2$ loop, thereby decreasing the thermostability by 6.3 °C. The F50A mutation within the hydrophobic cavity also lowered the $T_m$ by 9.7 °C. In addition, as expected, the $T_m$ of the F54A mutant decreased dramatically (81.8 °C), implying that the aromatic ring is crucial to the formation of the tight hydrophobic triad. Moreover, substitutions of hydrophobic core residues such as V11, I15, I72, and V73 with Ala also markedly dropped the $T_m$, revealing their essential roles in the tight hydrophobic packing of $Tm$-ACP. In particular, the $T_m$ of I15A dropped significantly (84.6 °C).

### 2.4. Hydrogen/Deuterium (H/D) Exchange Experiments

To explore the protection of amide protons and the local unfolding of hyperthermophilic $Tm$-ACP, the free energies of local unfolding ($\Delta G_{local}$) in $Tm$-ACP were determined by H/D exchange experiments. For slowly exchanged amide protons, whose peaks are shown on the first $^1$H-$^15$N HSQC spectrum after a 10 min exchange, the exchange rate constants ($k_{HDX}$) were determined from the decay of each peak height over a 1000 min exchange [44]. In cases in which the amide protons are exchanged by an EX2 mechanism [45], $k_{HDX}$ approximates to $K_{unfold} \times k_{rc}$ in the base-catalyzed regime of pD 5–7 [46], where $K_{unfold}$ is the equilibrium constant of the local unfolding reaction and $k_{rc}$ is the pD-dependent exchange rate for the random coil conformation. Because $k_{HDX}$ is determined by $K_{unfold}$ and $k_{rc}$, its value depends highly on pD. Within the range of pD 5–7, the log($k_{HDX}$) of amide protons increases by one unit as pD increases by one unit [44]. In contrast, $k_{HDX}$ in an EX1 mechanism is only characterized by...
the local unfolding rate constant ($k_{\text{HDX}} = k_{\text{unfold}}$), and pD has no effect on $k_{\text{HDX}}$. To confirm whether the exchange reaction of amide protons in $Tm$-ACP followed the EX2 mechanism, the pD dependences of $k_{\text{HDX}}$ of amide protons were compared at pD 5.5 and pD 6.5. As shown in Figure 3, all slowly exchanged amide protons in $Tm$-ACP behaved in accordance with the EX2 limit, implying that their exchanges were generally based on the EX2 mechanism. Because the equilibrium constant of the local unfolding reaction ($K_{\text{unfold}}$) could be approximated by $k_{\text{HDX}} / k_{\text{rc}}$, their free energies of local unfolding ($\Delta G_{\text{local}}$) could be determined by Equation (1) [46],

$$\Delta G_{\text{local}} = -RT \ln(K_{\text{unfold}})$$

where R is the universal gas constant, and T is the experimental temperature (25 °C). As expected, the amide protons of $Tm$-ACP were highly protected against the H/D exchange reaction, except for those in regions near helix III (Figure 4a). Ten minutes after the addition of D$_2$O, 46 amide peaks survived. The peaks of eight residues, V11, K12, I15, V69, I72, V73, I76, and E77, remained even after 1 month, indicating that these local regions required large energies to be unfolded. Surprisingly, the peak intensity of I15 was maintained at a nearly constant level for 1 month, making it impossible to determine its $k_{\text{HDX}}$ and $\Delta G_{\text{local}}$ from the H/D exchange experiment. This suggested that the amide proton of I15 could only be exposed to the solvent if the tertiary structure of the protein was completely denatured under a scenario of global unfolding.

![Figure 3. Log-log plot of the exchange rate constants ($k_{\text{HDX}}$) of amide protons in holo $Tm$-ACP measured at pD 5.5 and pD 6.5 at 25 °C. All amide protons that had computable $k_{\text{HDX}}$ values were included. The solid line represents the EX2 limit, log($k_{\text{HDX}, \ pD \ 5.5}$) = log($k_{\text{HDX}, \ pD \ 6.5}$) + 1, and the dotted line represents the EX1 limit, log($k_{\text{HDX}, \ pD \ 5.5}$) = log($k_{\text{HDX}, \ pD \ 6.5}$).](image)

To verify the importance of I15, we mutated I15 into an Ala residue. The H/D exchange experiment showed that weakened hydrophobic packing due to the I15A mutation reduced the free energies for the local unfolding of $Tm$-ACP (Figure 4b), demonstrating a critical role of this highly protected residue in the folding process. Compared with wild-type $Tm$-ACP, the I15A mutant protein had fewer amide peaks remaining on the first spectrum. The amide peaks of eight residues (Q25, D35, G37, A38, D39, M48, E53, and K57) in I15A completely disappeared after 10 min, which were retained for several hours in the wild-type protein. Furthermore, the amide protons of the core hydrophobic residues also showed faster exchange than those in the wild-type protein. Only the peaks of seven residues (V11, A15, V69, I72, V73, I76, and E77) remained after 1000 min. Similar to wild-type $Tm$-ACP, these peaks could be observed even after 1 month, but with much weaker intensities.
wild-type Tm-ACP, these peaks could be observed even after 1 month, but with much weaker intensities. For the wild-type protein, there were sixteen residues, V11, K12, I14, I15, S16, A30, K31, V47, F50, V69, G70, V73, S74, Y75, I76, and E77, which had large $\Delta G_{local}$ (>5kcal/mol) since their amide protons were located near the compact hydrophobic packing mediated by I15 (Figure 4c). Compared to this, replacement of A15 for I15 in the I15A mutant weakened the overall hydrophobic packing. As a result, two residues at the helix II (V47 and F50) and three residues at the helix IV (V73, I76, and E77) had decreased $\Delta G_{local}$ values (yellow spheres). This implies that the size and hydrophobicity of the side chain of I15 is important for maintaining the tight packing in the structure of Tm-ACP.

Figure 4. H/D exchange and Gdn-HCl induced unfolding experiments of Tm-ACP. Free energies of local unfolding ($\Delta G_{local}$) of amide protons in (a) wild-type and (b) I15A mutant holo Tm-ACPs from H/D exchange experiments. Red solid lines in the free energy of local unfolding plots indicate the respective free energy of global unfolding ($\Delta G_{global}$) at 25 °C. Black dashed lines indicate the lower limit of the global unfolding regime, [0.85]$\Delta G_{global}$. Decay curves of normalized peak intensities for the core hydrophobic packing residues are indicated as a function of time after the addition of D$_2$O. (c) The amide protons of sixteen residues with $\Delta G_{local}$ > 5kcal/mol were shown as spheres on the structure of Tm-ACP. As the replacement of A15 for I15 in the I15A mutant weakened the overall hydrophobic packing, two residues at the helix II (V47 and F50) and three residues at the helix IV (V73, I76, and E77), showed decreased $\Delta G_{local}$ values. The amide protons of those five residues were denoted in yellow. Gdn-HCl induced global unfolding of (d) wild-type and (e) I15A mutant holo Tm-ACPs was observed by monitoring the mean residue ellipticity at 222 nm with different concentrations of Gdn-HCl. Before the measurements, all samples were kept at 25 °C for 12 h to achieve complete equilibration of denaturation processes.

For the wild-type protein, there were sixteen residues, V11, K12, I14, I15, S16, A30, K31, V47, F50, V69, G70, V73, S74, Y75, I76, and E77, which had large $\Delta G_{local}$ (> 5kcal/mol) since their amide protons
were located near the compact hydrophobic packing mediated by I15 (Figure 4c). Compared to this, replacement of A15 for I15 in the I15A mutant weakened the overall hydrophobic packing. As a result, two residues at the helix II (V47 and F50) and three residues at the helix IV (V73, I76, and E77) had decreased $\Delta G_{local}$ values (yellow spheres). This implies that the size and hydrophobicity of the side chain of I15 is important for maintaining the tight packing in the structure of Tm-ACP.

2.5. Chemical Denaturation of Tm-ACP

To measure the stability of Tm-ACP upon chemical denaturation and confirm the importance of I15 in protein folding, we determined the free energies of global unfolding ($\Delta G_{global}$) of wild-type Tm-ACP and the I15A mutant using far-UV CD experiments [47]. The CD data were analyzed using a reversible two-state model for the native (N)-to-denatured (U) equilibrium and the linear extrapolation model shown in Equation (2) [47–50],

$$\Delta G_{global, [Gdn-HCl]} = \Delta G_{global} - m[Gdn-HCl]$$

(2)

where $\Delta G_{global, [Gdn-HCl]}$ is the free energy of global unfolding at a certain Gdn-HCl concentration ([Gdn-HCl]), $\Delta G_{global}$ is the free energy of global unfolding at a zero-denaturant concentration, and m is the slope of the fitted plot. $\Delta G_{global, [Gdn-HCl]}$ was calculated by fitting the data to equations (3) and (4).

$$K_{global, [Gdn-HCl]} = f_U / f_N = (1 - f_N) / f_N$$

(3)

$$G_{global, [Gdn-HCl]} = -RT \ln(K_{global, [Gdn-HCl]})$$

(4)

The fraction of the native state ($f_N$) was obtained from the resulting mean residue ellipticity values detected at 222 nm, and that of the unfolded state ($f_U$) was calculated as the sum of all fractions ($f_N + f_U$), which is always equal to one for a reversible two-state equilibrium. The equilibrium constant of the global unfolding reaction at a certain Gdn-HCl concentration ($K_{global, [Gdn-HCl]}$) was then calculated using Equation (3) [48,49]. Finally, $\Delta G_{global}$ was determined by rearranging Equation (2) to Equation (5) and substituting the Gdn-HCl concentration into the measured mid-point concentration ([Gdn-HCl]$_{1/2}$) [46,47,50].

$$\Delta G_{global} = m[Gdn-HCl]_{1/2}$$

(5)

Because the free energy required for local unfolding cannot exceed that required for global unfolding, we indirectly deduced the $k_{HDX}$ and $\Delta G_{local}$ values of hyperprotected I15 by approximating $\Delta G_{local}$ to $\Delta G_{global}$. Notably, the hyperthermostable Tm-ACP was also found to be exceptionally stable against Gdn-HCl-induced denaturation with an unusually high mid-point concentration ([Gdn-HCl]$_{1/2}$) of 4.58 M (Figure 4d), resulting in a $\Delta G_{global}$ value of 8.47 kcal mol$^{-1}$. Similar to the results for thermal denaturation, the I15A mutation destabilized the structure of Tm-ACP, substantially lowering [Gdn-HCl]$_{1/2}$ and $\Delta G_{global}$ to 2.88 M and 5.18 kcal mol$^{-1}$, respectively (Figure 4e). This implies that the hydrophobic side chain of I15 is a key factor for the global folding process of Tm-ACP.

3. Discussion

Hyperthermophilic proteins are more rigid than their mesophilic counterparts, and this structural rigidity is commonly considered as a prerequisite for high thermostability [51–54]. Salt bridges have been proposed to play a crucial role in increasing the rigidity and thermostability of hyperthermophilic proteins [29,30]. In addition to ionic interactions, hydrophobic interactions have also been reported to provide additional stabilization to the structures of thermophilic proteins [55,56]. In this study, we found that the ACP from a hyperthermophile, T. maritima, had extensive noncovalent interactions and thus an extremely thermostable structure with a melting temperature of 101.4$^\circ$C. Compared to mesophilic ACPs, the hyperthermophilic Tm-ACP has additional positive charges on its surface (Figure 1a, Figure 5). These positive charges not only neutralize the destructive repulsions between nearby negative charges, but also mediate extensive ionic interactions at the exterior surface of Tm-ACP,
stabilizing the overall structure. Moreover, Tm-ACP is the first ACP shown to harbor hyperprotected amide protons, requiring large free energies to be unfolded. This suggested that in addition to the compact hydrophobic interactions with I15, the distinct stabilization factors near the amide proton of I15 provided extraordinarily high rigidity at this local region, leading to hyperprotection.

Local unfolding events with ∆G_{local} close to ∆G_{global} may be identical to the global unfolding process [46]. To identify the amide sites that are unfolded only by the global unfolding process, each ∆G_{local} from H/D exchange experiments was compared with the ∆G_{global} value obtained from chemical denaturation experiments. For accurate comparison, chemical denaturation experiments were performed under the same buffer and temperature conditions that were used in the H/D exchange experiments. Similar to the results of Laity et al. [46], we defined a ∆G_{local} higher than 85% of ∆G_{global} (∆G_{local} > 0.85∆G_{global}) as identical to ∆G_{global}, considering the uncertainties of the measurements. The amide sites of I15 in helix I and two residues in helix IV, Y75 and I76, were found in the global unfolding regime (∆G_{local} > 0.85∆G_{global}) for the wild-type Tm-ACP at 25 °C. This implies that the hydrophobic side chain of I15 mediated tight hydrophobic packing between helix I and IV and that this packing may act as the last energy barrier of the global unfolding process of Tm-ACP. Because the folding reaction of Tm-ACP is reversible, the hydrophobic packing mediated by I15 may drive tertiary folding at very early steps by promoting hydrophobic packing among helix I, helix IV, and the α1α2 loop.

As shown in Figure S3, the I15A mutation caused large chemical shift perturbation in most of residues, implying that the replacement of A15 for I15 in the I15A mutant made the hydrophobic packing looser, and thereby, might cause significant change in the conformation of Tm-ACP. Although the amide proton of A15 in the I15A mutant was no longer as well-protected as I15 in wild-type Tm-ACP, it still played a role in the global unfolding process given the dramatic decrease in ∆G_{global}. In addition, the ^1H,^15N HSQC spectrum of the heat-treated I15A mutant also completely recovered (Figure S1b), implying that the mutation did not affect the reversibility of the folding reaction of Tm-ACP. These results indicate that the small hydrophobic side chain of A15 was also able to mediate the hydrophobic packing essential for the folding, but significantly lowered the energy barrier toward

![image](image-url)

**Figure 5.** Distributions of the surface charges in bacterial ACPs. All coordinates were obtained from Protein Data Bank (PDB) [15,23,38]. For all ACPs, negatively charged Glu and Asp residues are indicated in red and positively charged Arg and Lys residues are shown in blue. His residues (green) can also provide a positive charge in a physiological pH range. Only the hyperthermophilic Tm-ACP has extensive ionic interactions on its surface.
the unfolded state. Therefore, the proper size of the hydrophobic side chain at this conserved Ile site is critical for tight hydrophobic packing to promote the folded states of ACPs.

Bacterial cell membranes mostly consist of fatty acyl chains and have their own phase-transition temperatures resulting from variations in the lengths, degrees of saturation, and compositions of the acyl chains [33]. The hyperthermophile *T. maritima* possesses numerous long fatty acids, which comprise the thermostable cell membrane of the organism [32]. Therefore, *Tm*-ACP should be able to accommodate longer acyl chains than other mesophilic ACPs. In the crystal structure of mesophilic *Escherichia coli* ACP (Ec-ACP), the T39, A59, and T63 residues were revealed as the three outermost residues that form the entrance and interact with the prosthetic group [57]. The distances among these residues in holo Ec-ACP were 7.0 Å, 5.5 Å, and 7.6 Å, respectively (Figure 6a). For hyperthermophilic *Tm*-ACP, the distances between the Cα atoms of the corresponding residues, L43, L63, and S67, were increased to 8.6 Å, 7.5 Å, and 9.1 Å, respectively (Figure 6b), forming a much larger entrance. This would facilitate the entrance of long acyl chains into the hydrophobic pocket of *Tm*-ACP.

**Figure 6.** Structural comparison of bacterial ACPs. The top row depicts the size of each pocket entrance of (a) *Tm*-ACP (PDB ID: 6LVT), (b) *Ec*-ACP (PDB ID: 2K93) [38], and (c) *Ef*-ACP (PDB ID: 2N50) [18]. The size of the entrances was indicated as the distances between the three outermost residues that form each entrance. The middle row shows the distances between three helices, I, II, and III, of each protein. The bottom row displays the detectable cavities within the ACPs.

An atypical ACP (*Ef*-ACP) was found to be expressed as an auxiliary ACP that acts as a carrier for de novo fatty acid synthesis. After incorporating fatty acids from the host, these exogenous fatty acids are loaded to the atypical *Ef*-ACP and shuttled to the canonical FAS enzymes [58]. Since the exogenous acyl chains are frequently found to be long and unsaturated like oleic acid [58], *Ef*-ACP should have a large hydrophobic pocket to accommodate those long acyl cargos. In this respect, helices I and III of *Ef*-ACP were revealed to be protruded away from helix II, making an expanded space within the hydrophobic pocket [18]. The distances between three helices, I, II, and III, are greater in *Ef*-ACP than those in *Ec*-ACP (Figure 6c). Similarly, the hyperthermophilic *Tm*-ACP also showed
increased distances, thereby forming an expanded binding pocket. Therefore, along with the wide entrance, the expansion of the pocket seems to be crucial for the function of Tm-ACP in shuttling long acyl chains for the thermal adaptation of the T. maritima cell membrane.

4. Materials and Methods

4.1. Cloning, Expression, Isotopic Enrichment, and Purification

The acpP gene of T. maritima MSB8 was first cloned into the multi-cloning site of pET-11a vector by using two restriction enzymes, Ndel (catatg) and BamHI (ggatcc). The recombinant pET-11a vector was transformed into E. coli BL21 (DE3) [18]. To express isotope-labeled proteins, pre-cultured recombinant cells were inoculated into 500 mL of M9 minimal medium containing 50 mg/L ampicillin and isotopically enriched $^{15}$NH$_4$Cl and $^{13}$C-glucose (Cambridge Isotope Laboratories, Andover, MA, USA). After the optical density at a wavelength of 600 nm (OD$_{600}$) reached to 0.8–1.0, 1mM isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to induce the overexpression of Tm-ACP. The medium was placed at 37 °C and incubated for 6 hours. Purification of Tm-ACP was achieved by using its physicochemical properties, the surface charge (HiTrap Q FF and Resource Q, GE Healthcare Bio-Sciences, Uppsala, Sweden) and the molecular size (Superdex 75 16/600, GE Healthcare Life Sciences, Uppsala, Sweden) [18]. Usually, 5–10 mg of protein was yielded from 1 L of culture. After purification as the apo protein, Tm-ACP was converted to its holo form by using holo-ACP synthase from E. coli (Ec-AcpS) and coenzyme A (CoA) at 25 °C for 12 h [18]. The 25 mM Tris-HCl (pH 8) buffer with 20 mM MgCl$_2$ was used as a reaction buffer.

4.2. Site-Directed Mutagenesis

All mutation processes for Tm-ACP mutants were performed by polymerase chain reaction (PCR) amplification using various mutagenetic primer pairs (Appendix A Table A1). 10 fM of the pET-11a vector which contains the wild-type acpP gene of T. maritima MSB8 was used as a template. Each primer was used at a final concentration of 0.2 µM. The building blocks (dATP, dGTP, dTTP, and dCTP; 0.2 mM each) and 2 µM of nPfu-Forte DNA polymerase were added. After 30 cycles of denaturation (94 °C, 1 min), annealing (60 °C, 1 min), and elongation (72 °C, 5 min), the amplified vectors were transformed into E. coli BL21 (DE3). All mutant proteins were expressed and purified just the same as the wild-type protein.

4.3. NMR Experiments and Assignments

NMR spectroscopy experiments were performed using the Bruker Avance 700, 800 and 900 MHz spectrometers at the Korea Basic Science Institute (Ochang, Korea). 0.4–0.5 mM of the ACP samples was prepared in 330 µL of 9:1 (v/v) H$_2$O/D$_2$O 25 mM 2-(N-morpholino)ethanesulfonic acid (MES) buffer (pH 6.1) containing 5 mM CaCl$_2$ and 5 mM dithiothreitol (DTT). 2,2-dimethyl-2-silapentane-5-sulfonate (DSS) was used as an internal chemical shift reference. 0.02% of NaN$_3$ was added as an antiseptic. Triple resonance spectra of HNCO, HNCACB, and CBCA(CO)NH experiments were acquired to assign the resonances of spins within the backbones of Tm-ACP. For side chain assignment, CC(CO)NH, HBHA(CO)NH, H(CCO)NH, and HCCH-TOCSY spectra were obtained. The assignments were confirmed by $^1$H-$^1$N-$^1$H and $^1$H-$^{13}$C-$^1$H NOESY-HSQC spectra [18,59–62]. All NMR spectra were processed with NMRPipe [63] and analyzed with NMRFAM-Sparky [64]. Residual dipolar coupling (RDC) constants between two spins in backbone amide N-H bonds were determined by comparing spatially anisotropic dipolar couplings in IPAP-HSQC spectra of solution and gel phase Tm-ACP sample. The gel phase sample was prepared by dissolving the solution sample in a radially compressed polyacrylamide gel [18,62,65–67].
4.4. Solution Structure Calculation

Nuclear Overhauser effect (NOE) assignments were carried out using NMRFAM-Sparky [64], and the 3D structure of holo Tm-ACP was determined using Xplor-NIH-based calculations in the PONDEROSA-C/S package [39]. Thereafter, 20 lowest-energy structures were determined. All angle and distance violations, of the best 20 structures were analyzed and refined using PONDEROSA-Analyzer [40]. The final 20 lowest-energy structures were evaluated using PSVS [41]. Figures for the protein structures were generated using PyMOL (http://www.pymol.org). Final coordinates and NOE constraints have been deposited in the Protein Data Bank (PDB) under the accession number 6LVT (BMRB ID: 36242).

4.5. Hydrogen/Deuterium Exchange Experiments

The 15N-labeled Tm-ACP samples (0.5 mM) prepared for the NMR experiment were lyophilized for the H/D exchange experiment. D2O (100%) was added right before the experiments, and the successive HSQC data were collected every 10 min using two scans and time domains of 1000/256 for 1000 min at 25 °C. Additional HSQC spectra were acquired after 3, 7, 14, 21, and 28 days to monitor the surviving cross peaks. Each exchange rate constant ($k_{\text{HDX}}$) of amide protons was determined from the decay of peak height over time. For amide protons, which are exchanged by an EX2 mechanism [45], the exchange rate ($k_{\text{HDX}}$) approximated by $k_{\text{HDX}} = k_{\text{unfold}} \times k_{\text{rc}}$ in the base-catalyzed regime of pD 5–7, where $k_{\text{unfold}}$ is the equilibrium constant of the local unfolding reaction ($k_{\text{unfold}} = k_{\text{unfold}} / k_{\text{fold}}$) and $k_{\text{rc}}$ is the exchange rate for the random coil conformation [46,68]. Thus, $k_{\text{unfold}}$ approximates to $k_{\text{HDX}} / k_{\text{rc}}$. To acquire $k_{\text{rc}}$ for individual amide protons in the protein, $k_{\text{rc}}$ values for poly-\(\text{dL}\)-alanine at 293K (20 °C) as a function of pD [69] were first corrected by taking into consideration the inductive and steric effects of neighboring side chains [44]. Then, $k_{\text{rc}}$ values at 25 °C were calculated using the equation $k_{\text{HDX,T}} = k_{\text{HDX,293K}} \exp(-E_a(1/T – 1/293))$, where the temperature T is 298 K (25 °C) and $E_a$ is the activation energy [44]. The local unfolding energies ($\Delta G_{\text{local}}$) were also determined from $k_{\text{unfold}}$ [46].

4.6. Circular Dichroism Experiment

Secondary structure of Tm-ACP at various temperatures was assessed by Far-UV CD experiment using a J810 spectropolarimeter (Jasco, Tokyo, Japan). Thirty μM of the protein was dissolved in 25 mM MES buffer (pH 6.1) containing 5 mM CaCl2 and 5 mM dithiothreitol (DTT) and placed in a cuvette with a 1 mm path length. CD spectra were measured from 200 to 250 nm at 0.1 nm intervals. Temperature was increased gradually from 25 to 100 °C. The thermal denaturation of the protein was observed by monitoring the change in the mean residue ellipticities ($\theta$) at 222 nm wavelength. $\theta$ was calculated as described previously [70,71].

4.7. Chemical Denaturation Experiment

The chemical denaturant-induced global unfolding of wild-type and mutant Tm-ACPs was also investigated using Far-UV CD experiments. In brief, 30 μM of the proteins were dissolved in 25 mM MES buffer (pH 6.1) containing 5 mM CaCl2 and 5 mM DTT, and then different concentrations of Gdn-HCl were added to the protein solutions. After incubation at 25 °C for 12 h, complete equilibrium was achieved for all samples. The CD spectra of the samples were measured from 215 to 250 nm at 0.1-nm intervals. The CD data were analyzed using a reversible two-state model for the native (N)-to-denatured (U) equilibrium and the linear extrapolation model (Equation (2)) [47–50]. $\Delta G_{\text{global, [Gdn-HCl]}}$ was then calculated by Equations (3) and (4) [48,49]. Finally, $\Delta G_{\text{global}}$ was determined by using equation (5) [46,47,50].
4.8. Differential Scanning Calorimetry

The melting temperatures of wild-type and mutant Tm-ACPs were measured by DSC using a NanoDSC system (TA instruments, New Castle, DE, USA). The protein samples were prepared at concentrations of 2 mg/mL in 20 mM potassium phosphate buffer (pH 7.0). After degassing for 10 min, the reference buffer and the protein samples were equilibrated at 50 °C for 10 min. The thermograms were recorded as the temperature was increased at a rate of 1 °C/min from 50 °C to 120 °C. During the measurements, the pressure was kept constant at 3 atm to prevent the phase transition of the solvent. After polynomial baseline corrections and two-state scaled curve fittings, individual component peaks were resolved from the complex profiles.

4.9. X-ray Crystallography

Apo Tm-ACP was crystallized by 0.2 M zinc acetate dihydrate with 18–24% polyethylene glycol (PEG) 3350 precipitant at 20 °C. All crystals were harvested in the same buffer with 20% ethylene glycol as a cryoprotectant and stored in liquid nitrogen until data collection. X-ray diffraction data were collected at the beamlines 5C and 7A of the Pohang Accelerator Laboratory (Pohang, Korea). Crystals were maintained at −173 °C during data collection to prevent radiation damage. Diffraction images were integrated and scaled using the HKL2000 program suite [72]. The tertiary structure of apo Tm-ACP was determined by molecular replacement using the coordinates of Aquifex aeolicus ACP (PDB ID: 2EHS) as a search model. The initial structure was built by PHENIX [73] and modified using the WinCoot.[74] Average B-factors of the protein, water, and ligands were calculated by MOLEMAN2 [43]. Final coordinates of apo Tm-ACP were deposited in Protein Data Bank (PDB) under accession number 6LVU.

5. Conclusions

Hyperthermophilic Tm-ACP maintains its structure even at extremely high temperatures to function as an acyl carrier in the FAS system of T. maritima. Therefore, it is valuable to study the structural properties of this protein for understanding the adaptation strategies of proteins toward the extremely hot environments. Here, we provided the first NMR structure of Tm-ACP and demonstrated that extensive electrostatic interactions and enhanced hydrophobic packings cumulatively stabilize the structure of Tm-ACP, allowing it to perform its carrier function in the FAS system at extremely high temperatures. An ionic cluster between helices III and IV mediated by the nonconserved D59 residue forces the outward protrusion of helix III, resulting in a wide entrance of the hydrophobic cavity to facilitate the accommodation of long acyl chains required for thermal adaptation of the cell membrane of T. maritima. Moreover, Tm-ACP is the first ACP proven to harbor a hyperprotected amide proton for I15, which is also identified to be a key residue involved in global folding of the protein. It mediates hydrophobic interactions between helix I, II, and IV, and thus, facilitates the folding process of Tm-ACP. Our new insights into the structural properties of the hyperthermophilic Tm-ACP may provide a molecular understanding of the adaptational strategies employed by the primitive hyperthermophile to withstand the extreme temperatures associated with hot ancient marine environments.

Supplementary Materials: Supplementary materials can be found at http://www.mdpi.com/1422-0067/21/7/2600/s1, Figure S1: Reversibility of the thermal denaturation of (a) the wild-type and (b) the I15A mutant holo Tm-ACP was confirmed by comparing the 1H–15N HSQC spectra of each protein before (blue) and after (red) heat-treatments. The annealing was performed by heating the native sample in a boiling water bath for 15 min and cooling down to room temperature, Figure S2: Superimposition of three structures: the solution structure of holo Tm-ACP (blue, PDB ID: 6LVU) with the lowest energy, the crystal structure of apo Tm-ACP (yellow, PDB ID: 6LVU) and the solution structure of holo Ec-ACP (grey, PDB ID: 2K93) [38]. The red arrows indicate the protrusion of helix III of Tm-ACP compared to that of Ec-ACP, Figure S3: 1H–15N HSQC spectral overlay of the wild-type (black) and the I15A mutant (red) holo Tm-ACPs. The chemical shift perturbation of the eight residues, V11, L19, V26, L32, L36, L46, F50, and V69, whose sidechains contact closely with that of I15, were indicated as blue arrows.

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visualization, Y.L.; supervision, Y.K.; funding acquisition, Y.K. All authors have read and agreed to the published version of the manuscript.

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**Abbreviations**

| Abbreviation | Definition |
|--------------|------------|
| ACP          | Acyl carrier protein |
| ACPS         | Holo-ACP synthase |
| Bm           | Brucella melitensis |
| CD           | Circular dichroism |
| CoA          | Coenzyme A |
| DSC          | Differential scanning calorimetry |
| DSL          | Asp-Ser-Leu |
| DSS          | 2,2-Dimethyl-2-silapentane-5-sulfonate |
| DTT          | Dithiothreitol |
| Ec           | Escherichia coli |
| Ef           | Enterococcus faecalis |
| FAS          | Fatty acid synthesis |
| H/D          | Hydrogen/deuterium |
| HSQC         | Heteronuclear single quantum coherence spectroscopy |
| IPTG         | Isopropyl β-D-1-thiogalactopyranoside |
| NOE          | Nuclear Overhauser effect |
| OD           | Optical density |
| PCR          | Polymerase chain reaction |
| PDB          | Protein data bank |
| PEG          | Polyethylene glycol |
| Pt           | Pseudothermotoga thermarum |
| RDC          | Residual dipolar coupling |
| RMSD         | Root mean square deviation |
| RMSZ         | Root mean square of Z-scores |
| Ta           | Thermus aquaticus |
| Tm           | Thermotoga maritima |
| Vh           | Vibrio harveyi |

**Appendix A**

**Table A1.** Primers for the site-directed mutagenesis of *Tm*-ACP.

| Mutant | Direction | Primer Sequence |
|--------|-----------|-----------------|
| R4E    | forward   | 5′-gaa gga gat ata cat atg gcc agt gaa gaa att ttt tct aaa gtg aaa tc-3′ |
|        | reverse   | 5′-gat ttc act tta gaa aaa att tct tct tca ctg gcc ata tgt ata tct cct tc-3′ |
| K10E   | forward   | 5′-cgg gaa gaa att ttt tct gcc atg gaa tct tca gaa gaa at tct cct c-3′ |
|        | reverse   | 5′-gag atg atg gat ttc act tca gaa aaa at tct tcc cc-3′ |
| K12E   | forward   | 5′-gaa att ttt tct aaa gtg gaa tct atc atc tct gaa a-3′ |
|        | reverse   | 5′-ttc aga gat gga gga ttc cac ttt aga aaa aat ttt ctc-3′ |
| K18E   | forward   | 5′-cca tca tct ctg aag aat tgg ggg tgg tgg atg-3′ |
|        | reverse   | 5′-atg cca gcc ccc cca att ctt cgg gca aca tgg-3′ |
| K31E   | forward   | 5′-gag acg gaa gag gcc gaa tgg att gag gag ctc aag ggt gga g-3′ |
|        | reverse   | 5′-ctc cca gat cat caa tca atg cgg ctt cgg tca c-3′ |
| K57E   | forward   | 5′-gag atg tct gct gtc aag tgg atg cag cgg ccc-3′ |
|        | reverse   | 5′-ctc cct atg cat cca tca cag atg tgg cta aag gga gga g-3′ |
| K79E   | forward   | 5′-gct acg tct ccc atg cct ggt cgg cgc ccc-3′ |
|        | reverse   | 5′-gtt gct atg gag gac atg ctt cct cgg tca c-3′ |
| S16G   | forward   | 5′-gta aag tga atc tca tgg gga aat ccc ccc-3′ |
|        | reverse   | 5′-gta cta aag tga aat ccc cca tgg gga aat ccc ccc-3′ |
Table A1. Cont.

| Mutant | Direction | Primer Sequence |
|--------|-----------|-----------------|
| F8A    | reverse   | 5′-gat tca tcc acc ccc aat ttt tca ccg atg atg gat ttc act tta g-3′ |
|        | forward   | 5′-gcc agt cgg gaa gaa att gcc tct aaa gtt aac tcc tag-3′ |
| F50A   | reverse   | 5′-gac ctg atg gag ggg gaa agt gag tgc gg-3′ |
|        | forward   | 5′-ccg tca ctg tct gcc tgg tcc att acc agg tc-3′ |
| F54A   | reverse   | 5′-gga ctt tga gaa ggg ggg cgt taa tgg cag-3′ |
|        | forward   | 5′-cat cga ctt taa cgc cgg cct ccc tct cca agt cc-3′ |
| V11A   | reverse   | 5′-ccg gaa gaa att ttt tct aaa gcc aat ttc tct gaa aaa ttg g-3′ |
|        | forward   | 5′-cca att ttt cag aga tga tgg att tgg ctt tag aaa aat ccc g-3′ |
| I15A   | reverse   | 5′-cga ccc cca att ttt cag agg cga tgg att tca ctt tag aaa aat ccc-3′ |
|        | forward   | 5′-gaa att ttt ctt aaa gtt aac ttc atc gcc tct gaa aat ttc ggg gtc g-3′ |
| I72A   | reverse   | 5′-gaa ctt cta cgg cgg cgt cag cta cat tga aat ttt ccc-3′ |
|        | forward   | 5′-ctg tgg gcc gca tgg cag cta cat tga aat ttt ccc-3′ |
| V73A   | reverse   | 5′-ctg tgg gcc gca tgg cag cta cat tga aat ttt ccc-3′ |
|        | forward   | 5′-ctg tgg gcc gca tgg cag cta cat tga aat ttt ccc-3′ |

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