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Introduction of a thermophile-sourced ion pair network in the fourth beta/alpha unit of a psychophile-derived triosephosphate isomerase from *Methanococcoides burtonii* significantly increases its kinetic thermal stability

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ABSTRACT

Hyperthermophile proteins commonly have higher numbers of surface ionic interactions than homologous proteins from other domains of life. PfuTIM, a triosephosphate isomerase (TIM) from the hyperthermophile archaeon, *Pyrococcus furiosus*, contains an intricate network of 4 ion pairs in its 4th beta/alpha unit, (β/α)4, whereas MbuTIM, a triosephosphate isomerase from a psychrophile archaeon, *Methanococcoides burtonii*, lacks this network. Notably, (β/α)4 is the first element of the structure formed during folding of certain TIM-type (beta/alpha)8 barrel proteins. Previously, we have shown that elimination of PfuTIM's ion pair network in PfuTIM significantly decreases its kinetic structural stability. Here, we describe the reciprocal experiment in which this ion pair network is introduced into MbuTIM, to produce MutMbuTIM. Recombinant MbuTIM displays multi-state unfolding with apparent Tm values of autonomous structural elements approaching, or above, 70 °C, when a temperature scanning rate of 90 °C/h is used. The protein displays significant intrinsic kinetic stability, i.e., there is a marked temperature scan rate-dependence of the Tm values associated with unfolding transitions. The Tm values drop by as much as ~10 °C when the temperature scanning rate is lowered to 5 °C/h. MutMbuTIM, incorporating PfuTIM's ion pair network, shows significantly higher apparent Tm values (raised by 4–6 °C over those displayed by MbuTIM). MutMbuTIM also displays significantly higher kinetic thermal stability. Thus, it appears that the thermal stability of triosephosphate isomerase can be increased, or decreased, by either enhancing, or reducing, the strength of ion pair interactions stabilizing (β/α)4, presumably through reduced cooperativity (and increased autonomy) in unfolding transitions.

1. Introduction

1.1. Thermodynamic versus kinetic thermal stability

Proteins tend to withstand extreme (physical/chemical) environments that are structurally destabilizing in a manner based mainly upon whether they possess either (or both) of the following properties: (a) high thermodynamic stability, and (b) high kinetic stability. Proteins that are highly thermodynamically stable resist unfolding under extreme conditions essentially because the energy provided by the environment (e.g., high temperature) fails to shift the protein's conformational equilibrium from the native state into the unfolded state. When such proteins fail to display partial or complete unfolding at a given temperature, in theory, they can be relied upon to continue to resist undergoing unfolding at that temperature over infinitely long periods of incubation. Proteins that are not highly thermodynamically stable (but which have a high degree of kinetic stability) can also fail to show unfolding initially, just as is the case with highly thermodynamically-stable proteins; however, such proteins do gradually undergo thermal unfolding over extended periods of incubation at the given temperature. Therefore, in such cases, it is to be concluded that the environment does provide the energy equivalent of the difference in free energy between folded and unfolded states, although unfolding occurs extremely slowly, i.e., the protein displays kinetic stability. The attainment of conformational equilibrium in such 'kinetically stable' proteins from thermophile and hyperthermophile microbes appears to have become offset to such a high degree that populations of such proteins retain structure, and function, for inordinately long lengths of time [extending to years, or even centuries of time] under conditions that eventually prove to have been structurally destabilizing

Abbreviations: MbuTIM, *Methanococcoides burtonii* triosephosphate isomerase; MutMbuTIM, Mutant *Methanococcoides burtonii* triosephosphate isomerase; PfuTIM, *Pyrococcus furiosus* triosephosphate isomerase; MutPfuTIM, Mutant *Pyrococcus furiosus* triosephosphate isomerase; MALDI-TOF, Matrix-assisted laser desorption-ionization time-of-flight; MRE, Mean residue ellipticity; T_m, Temperature of melting; CD, Circular dichroism; DSC, Differential scanning calorimetry

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from a purely thermodynamic viewpoint. Numerous instances are now known of proteins that unfold inordinately slowly, even though the free energy change associated with their unfolding is only 5–15 kcal/mol [1–4]. It may be only a matter of time before the known numbers of such proteins increases to much larger numbers, since more and more proteins from thermophiles and hyperthermophiles are being produced and studied. It is impossible to overemphasize the point that high kinetic thermal stability is not true stability in thermodynamic terms. It is merely an 'operational' or 'functional' stability, since its existence allows proteins to retain structure(s) and function(s) for long durations of exposure to environmental conditions that eventually cause unfolding [5–10].

From a purely functional point of view, however, high kinetic stability must be thought to be at least as important as (if not even more important than) high thermodynamic stability, since naturally-occurring proteins are mostly required to survive and function for a limited duration of a few minutes or hours [11] and not for ever. If a protein is folded into a sufficiently kinetically-stable structure, it can resist unfolding and retain full functionality for the entire duration of its normal/designated existence *in vivo* without needing to either: (a) be highly thermodynamically stable, or (b) even be folded into the most stable of all structures conceivable for that protein. Therefore, understanding the basis of kinetic stability in proteins is critical to the development of applications allowing proteins to be stabilized or destabilized by design using rational protein engineering-based methods [12–15].

1.2. The kinetic stability of proteins from extremophile organisms

Proteins from extremophile organisms tend to be both thermodynamically as well as highly kinetically stable. Increasingly, it appears that it is the high kinetic stability of such proteins that is primarily responsible for their inordinately high structural stability. Measurements of the thermodynamic stabilities of hyperthermophile proteins at their normal temperatures of functioning, under conditions of conformational equilibrium, suggest that the free energy changes associated with their unfolding at 'hyperthermophile' temperatures are only marginally higher than that of analogous mesophile homologs at 'mesophile' temperatures [1–4]. More specifically, if the change associated with unfolding is not of the order of ~5–15 kcal/mol (as it tends to be for mesophile proteins), it tends to be somewhat higher, at 20–25 kcal/mol, but generally no higher. In contrast, the kinetic stabilities of mesophile and hyperthermophile homologs are very different. For conditions that initiate unfolding, it is well-known that unfolding rates are dependent on temperature, or denaturant concentration, varying over one or two orders of magnitude [16,17]. In contrast, thermophile proteins are generally found to attain conformational equilibrium over timescales that are several orders of magnitude longer than those applicable to unfolding of mesophile homologs [5–10,18].

1.3. A hypothesis concerning kinetic thermal stability

For several years now, our group has been interested in understanding and manipulating kinetic stability in proteins. Our contention is that a protein's kinetic thermal stability depends mainly on the autonomy of structural stabilization of component sub-structures (e.g. helices, sheets, or combinations of such structures, forming supersecondary structural elements) within the protein, with the number and nature of such sub-structures determining the level of cooperativity that is seen in the overall (global) unfolding process for the entire protein's structure [19–25]. More specifically, our contention is that if a protein's sub-structures tend to be reasonably autonomously stable, they would not be expected to be dependent on the energy of stabilization gained through packing interactions with neighboring sub-structures for formation (or retention) of structure. Consequently, structural unfolding would tend to be non-cooperative at the global level. In other words, if a particular sub-structure in a hyperthermophile protein were to undergo unfolding, this would have little effect on neighboring sub-structures. Therefore, no cooperative global unfolding of the protein's structure would occur as a consequence of the unfolding of any individual sub-structure. The non-occurrence of a cooperative global unfolding transition would afford each particular sub-structure undergoing unfolding the opportunity to refold and re-associate with its neighboring sub-structure(s). Thus, each unfolding event involving a sub-structure would be unproductive in respect of its influence on global unfolding, frustrating the unfolding process and effectively slowing it down. Our hypotheses, therefore, is that reduced cooperativity of global unfolding (achieved through increased autonomy of formation, and stabilization, of sub-structures) is one mechanism by which a protein can gain high kinetic stability, regardless of the exact details of the mechanisms by which individual sub-structures are stabilized.

1.4. Protein engineering of kinetic thermal stability in a TIM Barrel protein

In a recent paper [25], we have shown that mutational disruption of an elaborate ion pair network on the surface of a small element of sub-structure in *Pyrococcus furiosus* triosephosphate isomerase (PfuTIM) achieves a dramatic reduction in the protein's kinetic thermal (conformational) stability, causing it to transform from being an extraordinarily hyperthermostable protein into a protein of ordinary thermostability. In this particular case, the element of sub-structure happened to be a helix in the fourth beta/alpha unit, $(\beta/\alpha)_4$, of the eightfold beta/alpha barrel (TIM barrel) structure of PfuTIM. Notably, the first four β/α units and, in particular, the fourth β/α unit, $(\beta/\alpha)_4$, is thought to play a crucial role in the folding of proteins with the TIM barrel type of fold, with $(\beta/\alpha)_4$ being the first of the β/α units to form during folding [26,27]. The precise mutations that we introduced (Fig. 1A and B) were based on an examination of the analogous stretch of sequence in a related triosephosphate isomerase from a psychrophile organism, *Methanococcoides burtonii* (MbuTIM). In MbuTIM, no scope exists for the occurrence of analogous ion pair interactions within the corresponding helix. The entire sequences of the two proteins are aligned and shown in Supplementary Fig. 1.

1.5. The scope of the present work

In the present paper, our intention was initially to perform an experiment constituting the exact reverse of the previous experiment, i.e., we wished to introduce PfuTIM's ion pair network into MbuTIM to see whether this would dramatically improve the kinetic stability of this psychrophile/psychrotolerant protein. While there was indeed a significant enhancement of kinetic thermal stability in MbuTIM as a consequence of this engineering, we were surprised to discover that MbuTIM was naturally a reasonably intrinsically kinetically stable protein in its own right. Thus, rather than introducing some kinetic thermal stability into a protein that we had presumed would lack substantial kinetic stability a priori, we found MbuTIM to be substantially kinetically stable; however, we managed to significantly further improve both MbuTIM's kinetic stability and its thermodynamic stability by introducing PfuTIM's ion pair network into it.

1.6. The organism Methanococcoides burtonii and its proteins

A few words of introduction to the organism, *M. burtonii*, would be in order. *M. burtonii* is a cold-adapted archaeon from the methane-saturated, permanently-cold waters of a lake in Antarctica [28]. It grows naturally at a temperature of about 1 °C, but has a temperature range of growth of −2.5 to 28.0 °C, with optimum growth at 23.0 °C [29]. Recent work done to examine the mechanism of cold adaptation in this organism, through extensive studies of differences in lipid composition and global proteomic profiles during growth at different temperatures [30,31], has provided insights into how the organism manages to be psychrotolerant. Global comparative analyses of the sequences of its proteins and those of mesophile and thermophile homologs confirm that

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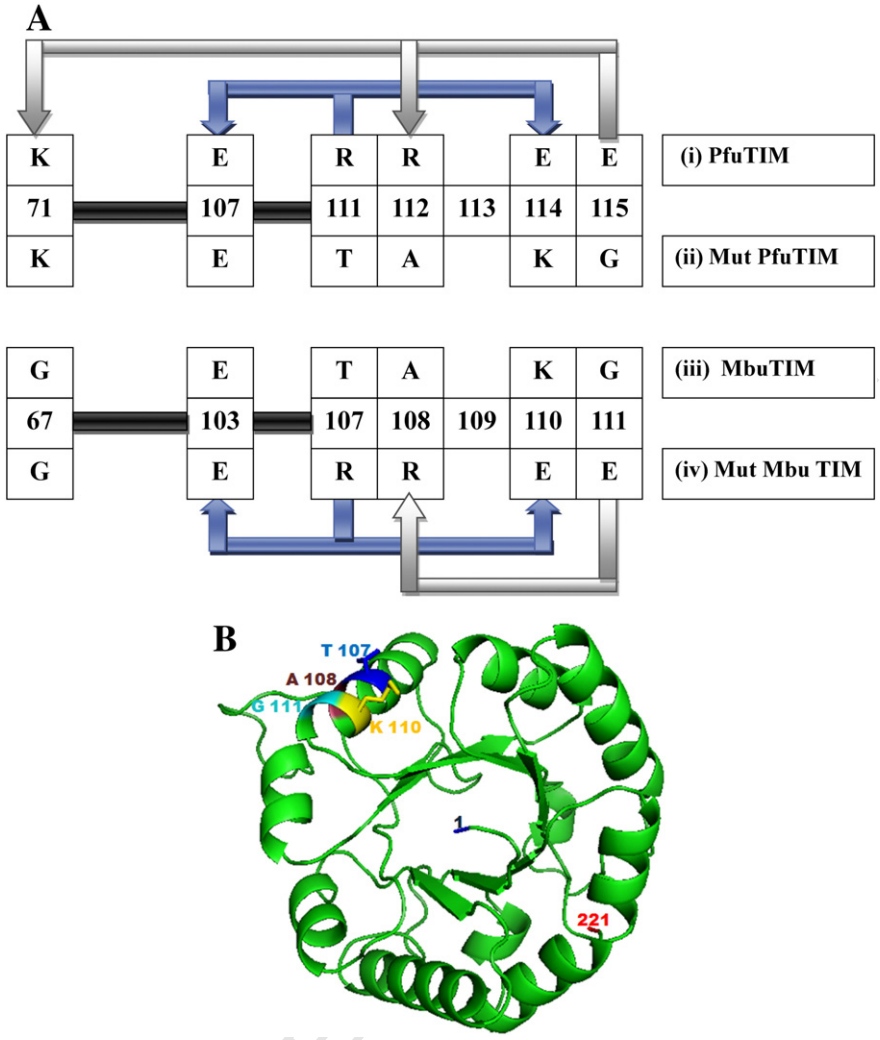

Fig. 1. (A) Charged residues and ion pair interactions in corresponding regions of the helices in $(\beta/\alpha)_4$ of PfuTIM and MbuTIM, shown along with the mutations introduced in PfuTIM in a previous study (25), and the mutations introduced into MbuTIM in this study. (i) PfuTIM: Ion pair interactions in wild type PfuTIM (Arg111 interacts with Glu107 and Glu114, Glu115 interacts with Arg112 and Lys71; note that Lys71 is not in the fourth beta/alpha unit). (ii) MutPfuTIM: Mutant PfuTIM incorporating residues from MbuTIM that destroy all ion pair interactions originally occurring in wild type PfuTIM. (iii) MbuTIM: Wild type MbuTIM sequence, showing residues at positions homologous to those of charged residues in wild type PfuTIM, with no scope for PfuTIM-like ion pair interactions. (iv) MutMbuTIM: Ion pair interaction possibilities introduced into MbuTIM, for this study, by replacing its own residues by those engaging in ion pair interactions in wild type PfuTIM at analogous positions (4 mutations were introduced: Thr107Arg, Ala108Arg, Lys110Glu, Gly111Glu to create scope for formation of three 3 ion pairs Arg107 with Glu103 and Glu110; Glu111 with Arg108). (B) A putative structure for MbuTIM showing the locations of the mutations made in $(\beta/\alpha)_4$. The structure was plotted using PYMOL, based on coordinates produced by the web-server running SWISS-MODEL.

the organism is a psychrophile, but not a psychrotroph, i.e., it grows optimally at 23 °C although it can grow at temperatures as low as −2.5 °C and as high as 28 °C [32]. It is generally found that the temperature of melting of proteins from *M. burtonii* tends to be somewhere between 40 and 50 °C [33–35].

2. Experimental

2.1. Chemicals and buffers

All chemicals were obtained from either GE Healthcare (USB Chemicals), USA, or Sigma Chemical Co. USA. Tris·HCl buffer of pH 7.5 was used.

2.2. Cloning, expression and purification of MbuTIM

The gene (UniProt accession code: Q12UK2, EMBL accession code: ABE52874.1.) encodes *M. burtonii* (DSM 6242) triosephosphate isomerase (MbuTIM). The gene's sequence, codon optimized for expression in *Escherichia coli*, was gotten synthesized through custom-synthesis by GenXBio, USA. The gene was then amplified using the following specific primers: Forward Primer 1: 5′-TGA GTC ACA TAT GAA ACC GCT GAT CGT TCT GAA CC-3′; Reverse Primer 1: 5′-TTC GAA TCT CGA GTT AGT GGT GGT GGT GGT GAT GGA TCAG AGA AAC CAG-3′. The resulting amplicon contained sites for NdeI at the 5′ end, and XhoI at the 3′ end, and a sequence encoding a 6xHis affinity tag encoded within the 3′ end of reverse primer, immediately before the stop codon preceding the XhoI site. The amplicon was digested and inserted between the NdeI and XhoI sites in the vector pET23a vector (Novagen). The insert-carrying plasmid was sequenced. This allowed us to verify that the gene had the correct DNA sequence. The plasmid was transformed into BL21(DE3)Star pLys *E. coli* strain for expression. Induced expression was obtained by overnight incubations of cultures using 1 mM IPTG. Protein was purified by lysing cells and performing Ni-NTA chromatography under non-denaturing conditions, with elution from the Ni-NTA column obtained using 250 mM imidazole.

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2.3. Structure of MbuTIM

The amino acid sequence of MbuTIM was sourced from the UniProt database (see above section) and supplied as input to the web-enabled software SWISS-MODEL (http://swissmodel.expasy.org) for structural homology-based modeling. The output from this server (Supplementary Fig. 2) included the coordinates of the modeled structure, which were used to produce the ribbon-diagram shown in Fig. 1B through use of the software PYMOL.

2.4. Cloning, expression and purification of MutMbuTIM

Using the gene encoding MbuTIM (please see above section) as initial template DNA, and the technique known as splicing by overlap extension polymerase chain reaction (SOE-PCR) to introduce mutations, we generated the gene, MutMbuTIM, encoding a variant of MbuTIM incorporating 4 mutations within a contiguous stretch of 5 amino acids, using the original primers from the termini of the MbuTIM gene, and the following two complementary (overlapping) primers spanning the region of the polypeptide containing the mutations: Forward Primer 2: 5′-AGG CGT CTG TTC GTC GTG CGG AAG AAG AAG GTC TGC GTA CCA TCA TCT GCA CCA ACA ACA TCG CG-3′; Reverse Primer 2: 5′-CGC AGA CCT TCT TCT TCC GCA CGA CGA ACA GAC GCC TCG ATG TCA GCA AGT TTC AGA CGG C-3′. Amplicons generated by separate PCR reactions using (i) Forward Primer 1 and Reverse Primer 2, and (ii) Forward Primer 2 and Reverse Primer 1, were further spliced and amplified by using the amplicons themselves as mega-primers and the original Forward Primer 1 and Reverse Primer 1. For all PCR reactions, the initial hot-start denaturation was done at 98 °C for 5 min, followed by 30–35 cycles of denaturation at 98 °C for 1 min, annealing at 55 °C for 2 min, and extension at 73 °C for 3 min.

The mutations introduced were designed to result in the following residue replacements in the expressed MutMbuTIM protein: Thr107Arg (T107R), Ala108Arg (A108R), Lys110Glu (K110E), Gly111Glu (G110E), respectively. Of the above, three mutations (T107R, A108R, and G111E) replace non-charged residues naturally present in *M. burtonii* TIM with the corresponding structurally analogous charged residues used by *P. furiosus* TIM. In addition, one mutation (K110E) replaced a charged residue naturally present in *M. burtonii* TIM with the corresponding structurally analogous residue of opposite charge used by *P. furiosus* TIM. The mutations were anticipated to effectively introduce three ion pair interactions within the structure of MbuTIM, where none are thought to have existed earlier.

Fig. 1A displays a comprehensive schematic summary of the various sets of analogous charged and non-charged residues in PfuTIM and MbuTIM, as well as their locations, differential identities and likely ion pair interactions in (i) PfuTIM, (ii) MutPfuTIM, (iii) MbuTIM, and (iv) MutMbuTIM. The gene encoding MutMbuTIM was cloned and expressed exactly as described above, for MbuTIM, and also sequenced to confirm that we had the correct DNA sequence. The mutant protein was also purified exactly as described above for MbuTIM.

2.5. Circular dichroism (CD) studies

Far-UV CD spectra were acquired on a JASCO J-810 spectropolarimeter using a cuvette of path length 2 mm, and a spectral bandpass of 4 nm. Raw ellipticity data were converted to mean residue ellipticity using the formula $[\theta] = [\theta_{raw} \times 100 \times MRW]/c \times l$, where MRW is the mean residue weight for MbuTIM (103 Da), c is the concentration of MbuTIM in mg/ml and l is the path length in cm. Changes in mean residue ellipticity (at 218 nm) as a function of time were monitored at a fixed set of temperatures (60 °C, 69 °C, 73 °C, 76 °C, 79 °C and 83 °C) for time scans involving both MbuTIM and MutMbuTIM. Changes in mean residue ellipticity (at 218 nm) as a function of temperature were monitored, for both MbuTIM and MutMbuTIM, using constant temperature ramping rates of 3 °C/min or 1 °C/min, using the spectropolarimeter's Peltier block arrangement (PTC-423S) and a cuvette of path length 1.0 cm.

2.6. Chromatographic and electrophoretic studies

Gel filtration chromatography was performed on a suitably equilibrated analytical Superdex-200 column using an AKTA Purifier-10 chromatographic workstation (GE-Pharmacia) for comparison of the hydrodynamic volumes of MbuTIM and MutMbuTIM. SDS-PAGE electrophoresis was carried out by standard methods.

2.7. Mass spectrometry (MS)

A voyager DE-STR MALDI-TOF mass spectrometer was used for determination of the intact mass of MbuTIM and MutMbuTIM and also for peptide mass fingerprinting-based confirmation of the identities of both proteins, based on digestions of both proteins by the enzyme trypsin according to standard protocols for in-gel tryptic digestion from SDS-PAGE bands.

2.8. Differential scanning calorimetry (DSC)

Initially, the structure-melting temperatures of MbuTIM and MutMbuTIM were analyzed through heating between 20 °C and 90 °C at rate of 1 °C/min on a Nano-DSC differential scanning calorimeter (TA-Waters, USA). Software available with the instrument was used for curve-fitting analysis to derive melting temperatures for the endothermic transitions observed. Subsequently, a VP-DSC instrument from MicroCal was used to examine the temperature scan-rate dependence of transitions in MbuTIM and MutMbuTIM, by performing DSC scans at one or more of the following temperature scan rates: 90 °C/h, 60 °C/h, 30 °C/h, 15 °C/h and 5 °C/h. These scans were performed both to examine whether either (or both) of the proteins undergo multi-state unfolding transitions (indicating the existence of different autonomously unfolding structural elements) and also to examine whether Tm values of various observed unfolding transitions tend to reduce with lowered temperature scan rates (indicating significant kinetic stabilization of the structures of these proteins). Certain DSC scans were also performed in the presence of 2 M NaCl, to examine the effect of altered salt concentrations on the Tm values of unfolding transitions.

2.9. Analytical ultracentrifugation (AUC)

Analytical ultracentrifugation studies with MbuTIM were performed on a Beckman Coulter Proteome Lab XL-I analytical ultracentrifuge equipped with UV optics to detect protein boundary migration during ultracentrifugation through monitoring of absorption at 280 nm through a quartz window within the sample cell, using a protein sample with an absorbance of 0.6 (amounting to a protein concentration of ~2.4 mg/ml). Data were analyzed using standard software.

2.10. Multi-angle light scattering (MALS)

MALS studies were performed using a Wyatt Technology Corporation Helios Dawn 8+ instrument, in cuvette mode, using static light scattering data from 8 angles of scattering, and an MbuTIM protein sample with a concentration of 2.4 mg/ml. Data were analyzed using standard software available for use with the instrument.

3. Results and discussion

To the best of our knowledge, the triosephosphate isomerase protein encoded by the genome of *M. burtonii* (MbuTIM) has never before been either purified from *M. burtonii*, or produced in recombinant form through heterologous overexpression in any organism. Thus, this paper

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reports the first set of biochemical/biophysical data for this protein, and for its mutants designed to alter the protein's kinetic thermal stability.

MutMbuTIM refers to a variant of MbuTIM which contains 4 mutations that introduce a network of 3 ion pairs into MbuTIM, where none existed hitherto. The details of the mutations (see Materials and methods) are reiterated once again below, for quick reference: T107R, A108R, K110E, and G111E. The mutations (effecting replacement of T by R, A by R, K by E, and G by E) dramatically enhance the scope for the occurrence of ion pair interactions. They are all based on residues found at structurally analogous positions in a hyperthermophile TIM, known as PfuTIM, which contains a rich network of ion pairs at this location, representing the helix component of the fourth beta/alpha unit, $(\beta/\alpha)_4$.

3.1. MbuTIM and MutMbuTIM are well-folded beta|alpha proteins with identical secondary structural contents

Fig. 2A and C shows SDS-PAGE data for the expression and purification of MbuTIM, and MutMbuTIM, respectively. Similarly, Fig. 2B and D shows confirmatory mass spectrometric data for the intact masses of these two proteins. Supplementary Figs. 3 and 4 additionally show peptide mass fingerprinting (PMF) data for MbuTIM, confirming that the expressed proteins are indeed MbuTIM, and MutMbuTIM, respectively. Supplementary Fig. 5 shows sequence coverage data for MbuTIM and MutMbuTIM, as well as the sequences of the 6 peptides each from MbuTIM and MutMbuTIM that are detected during PMF data collection. Notably, it is seen in Supplementary Fig. 5 that although five out of the six identity-confirming peptides detected from MbuTIM and MutMbuTIM tryptic digests are identical in sequence (and mass), a sixth peptide that derives from the region incorporating the ion pair-introducing mutations shows differential digestion patterns, suggesting changes in the region of the protein around the mutations that affect the digestion pattern.

The far-UV CD spectra of MbuTIM and MutMbuTIM are shown in Fig. 3A. The two spectra are virtually super-imposable, indicating that the mutations introduced in MbuTIM to create MutMbuTIM have no profound detectable effect on the protein's folded structure. Similarly, gel filtration chromatograms (Fig. 3B and C) indicative of the hydrodynamic volumes of the two proteins show that the mutations have had no effect on quaternary structure.

Interestingly, both proteins behave like proteins with masses of about ~160 kDa on a 24 ml Superdex-200 column (the calibration data for which may be found in Supplementary Fig. 10). The calculated subunit mass of MbuTIM is ~23 kDa, indicating that both MbuTIM and MutMbuTIM are hexameric in nature, during gel filtration under a hydrostatic pressure of approximately 0.5 MPa (about 5 atmospheres). However, the quaternary structure of MbuTIM at normal temperature and pressure turned out to be dimeric under conditions of examination by analytical ultracentrifugation (Supplementary Fig. 6) and multi-angle light scattering (Supplementary Fig. 7). This is possible to explain by referring to the fact of *M. burtonii* being a piezophilic psychrophile. Piezophile-derived proteins are adapted to high hydrostatic pressure and can tend to show subunit associations upon increase in hydrostatic pressure, unlike most ordinary proteins that show subunit dissociations with increase in pressure. For the moment, since most of the other biophysical-chemical studies (including DSC) for this paper have been performed under near normal conditions of pressure, we can probably

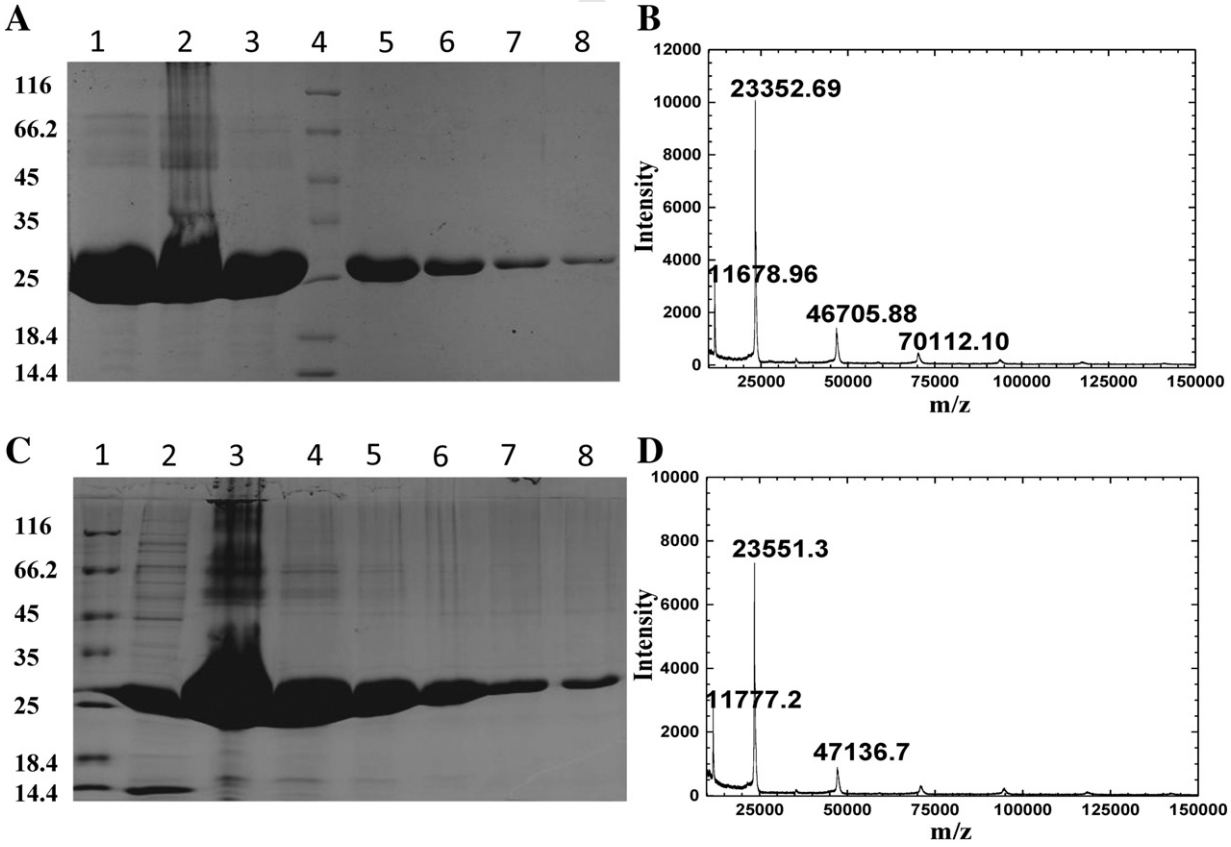

Fig. 2. SDS page profiles and MALDI-TOF spectra of intact forms of MbuTIM and MutMbuTIM. (A) SDS page profile showing MbuTIM purified under non-denaturing conditions using Ni-NTA chromatography and elution in 250 mM imidazole. Lanes 1–3 and 5–8 show 16 μl samples from successive 1.5 ml fractions (with molecular weight markers in lane 4); (B) MALDI-TOF spectrum of intact, purified MbuTIM; (C) SDS page profile showing MutMbuTIM purified under non-denaturing conditions using Ni-NTA chromatography and elution in 250 mM imidazole. Lanes 2–8 show 16 μl samples from successive 1.5 ml fractions (with molecular weight markers in lane 1); (D) MALDI-TOF spectrum of intact MutMbuTIM.

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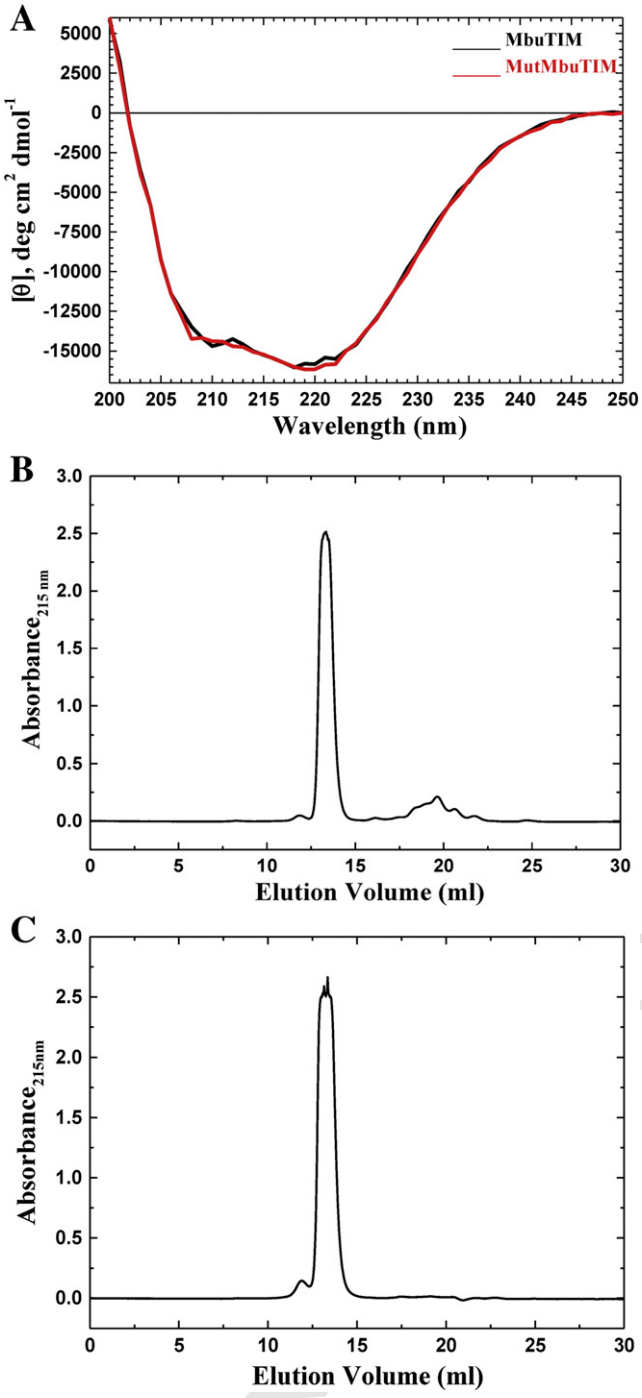

Fig. 3. Structural-biochemical comparisons of MbuTIM and MutMbuTIM. (A) Overlapping far-UV CD spectra of MbuTIM and MutMbuTIM; (B) Gel filtration (Superdex-200) chromatogram of MbuTIM, in 20 mM Tris buffer pH 7.5; (C) corresponding gel filtration (Superdex-200) chromatogram of MutMbuTIM.

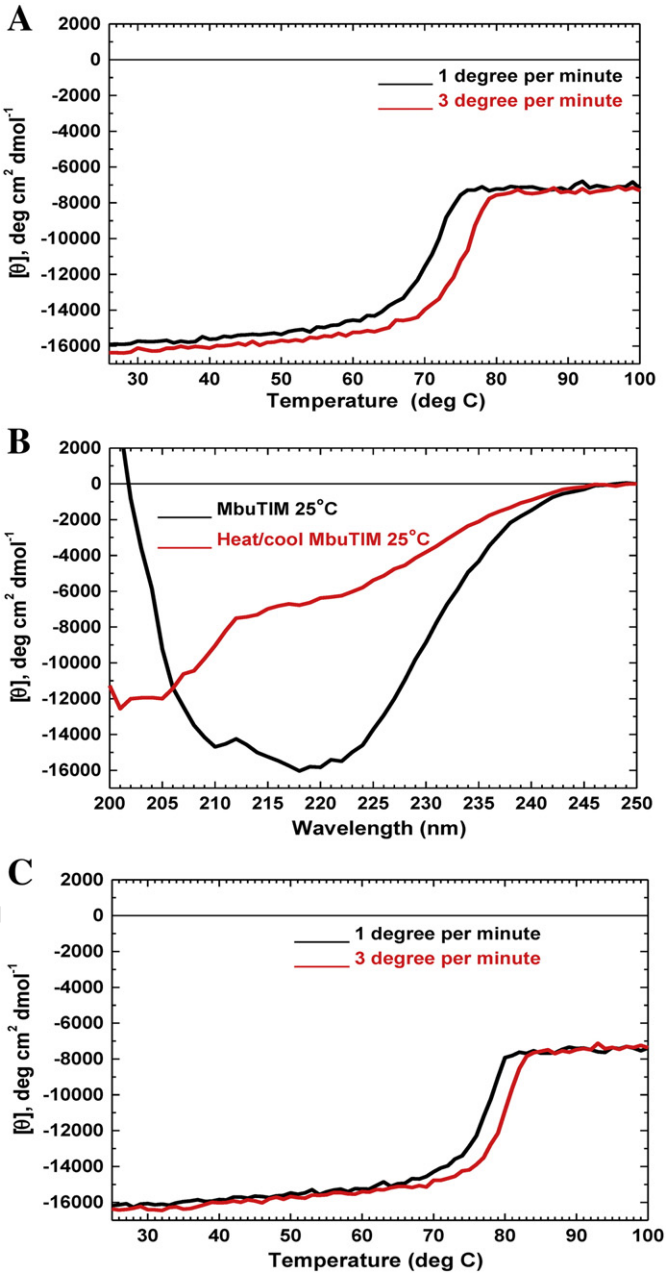

Fig. 4. Thermal melting of MbuTIM and MutMbuTIM. Temperature- and scan rate-dependence of thermal melting of MbuTIM (panel A), and MutMbuTIM (panel C) showing mean residue ellipticity changes at 218 nm for scan rates of 1 °C/min (black) and 3 °C/min (red); Comparison of the far-UV CD spectrum of MbuTIM (panel B) at 25 °C (black) with that of the same protein after heating to 100 °C and cooling back to 25 °C (red).

assume that MbuTIM is a dimer that shows a tendency to form hexamers through the trimeric assembly of dimers when there is an increase in hydrostatic pressure.

3.2. MbuTIM displays unanticipated High Thermal Stability

Fig. 4A shows data relating to the temperature- and time-dependence of loss of secondary structural content in MbuTIM during heating. The protein shows an unfolding curve with a midpoint (apparent T_m) of ~73 °C for a heating rate of 1 °C/min. When the rate of heating is increased to 3 °C/min, the apparent T_m is slightly higher, at about ~76 °C. These high values of apparent T_m suggest that MbuTIM is thermostable. This is entirely unanticipated, given that the protein is encoded by the genome of a psychrophile organism, *M. burtonii*. In fact, in studies of the thermostability of certain other proteins from *M. burtonii*, it has been observed that the T_m is usually between 40 and 50 °C. This makes the thermostability of MbuTIM even more surprising.

Further, it is also surprising that there is a heating rate-dependence of unfolding of MbuTIM, which is evident in the differences in the unfolding curves (and apparent T_m values) obtained through heating at 1 °C/min, and at 3 °C/min. This heating-rate dependence clearly indicates kinetic stability in MbuTIM, since proteins that are purely thermodynamically stabilized are not expected to show such behavior.

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Notably, Fig. 4A also shows that unfolding does not involve a complete loss of secondary structure. Rather, there is only about 50% loss of negative mean residue ellipticity, from −16000 to about −7000 deg cm^2 $dmol^{-1}$, suggesting that there is no global unfolding but rather only partial unfolding (although, in certain batches, a greater degree of unfolding was also observed). Cooling does not reverse this 50% loss of secondary structural content, indicating that the parts of the protein that unfold upon heating remain unfolded. This is also clearly seen in Fig. 4B which shows the far-UV CD spectra of MbuTIM collected (i) at 25 °C, before heating to 100 °C, and (ii) at 25 °C, following heating to 100 °C and cooling back to 30 °C. There is a clear loss of negative mean residue ellipticity between 205 nm and 240 nm, indicating loss of helical/sheet content, with increase in the negative mean residue ellipticity signal at ~200 nm, indicating enhanced content of either random coil structure or polyproline type II (PPII) structure.

The lack of observable refolding is consistent with the observed irreversibility of refolding seen with many proteins derived from extremophiles [36,37]. As the protein is not completely unfolded even at 100 °C, the unfolding data cannot be discussed classically, in part because of the non-two-state behavior and in part due to the apparent irreversibility of the unfolding transitions associated with partial-unfolding. Therefore, we decided to explore the protein further. Unfortunately, as the sequence contains no tryptophan (W) residue, there is no scope for monitoring tertiary structural transitions reliably, using either fluorescence spectroscopy, or near-UV CD spectroscopy. Both of these methods are critically dependent on the spectroscopic behavior of tryptophan; the fluorescence emission maxima of the other aromatic residues are not dependent on the polarity of the environment, and their mean residue ellipticity signals are too poor to allow tertiary structural monitoring through CD spectroscopy.

3.3. MutMbuTIM shows a substantially higher thermal stability than MbuTIM

Fig. 4C shows the temperature- and time-dependence of loss of secondary structural content in MutMbuTIM during heating. As seen with MbuTIM, the protein shows unfolding curves with different midpoints (apparent T_m values) for heating rates of 1 °C/min and 3 °C/min. Interestingly, both values of apparent T_m show similar increments in MutMbuTIM. The apparent T_m is ~78 °C for a heating rate of 1 °C/min, and ~80 °C for a heating rate of 3 °C/min; evidently, the introduction of three ionic interactions in the fourth beta\alpha unit of MbuTIM has resulted in a significant enhancement of its apparent melting temperature by almost 6 °C. The fact that there is a correspondence in the rise in apparent T_m for corresponding heating rates indicates that the effect of the mutations is primarily upon the kinetic aspects of the thermal stability of MbuTIM. Notably, with MutMbuTIM too, unfolding does not involve a complete loss of secondary structure. Rather, here too, there is only about 50% loss of negative mean residue ellipticity, from −16500 to about −7500 deg cm^2 $dmol^{-1}$, with no refolding seen upon cooling. Thus, in MutMbuTIM too there is no global unfolding.

3.4. The high kinetic thermal stability of MbuTIM is very significantly enhanced by the mutations introduced to create MutMbuTIM

MbuTIM's high kinetic thermal stability becomes further evident when the protein's unfolding rates at different temperatures are examined over a wide range of temperature (60 °C to 83 °C). Remarkably, the already high kinetic thermal stability of MbuTIM is profoundly enhanced through the introduction of the 4 mutations (facilitating 3 ion pair interactions) used to create MutMbuTIM.

Fig. 5A to F shows the rates of loss of structure in MbuTIM and MutMbuTIM as a function of time of incubation at temperatures of 60 °C, 69 °C, 73 °C, 76 °C, 79 °C and 83 °C. The first important observation is that both proteins undergo unfolding at all of these temperatures, with unfolding proceeding to the same extent (approximately 50% loss of structure) at most temperatures. Of course, in Fig. 5A which shows data for incubation at 60 °C, the degree of unfolding observed is lower for the duration of observation. However, we examined the protein(s) for much longer durations at these low temperatures separately and found that they all unfold to the same extent that is seen for incubation at higher temperatures. At 64 °C, MbuTIM takes 2 h to reach 50% unfolding, while at 60 °C it takes more than 4 h (Supplementary Fig. 11A, B). If MbuTIM's thermal stability had owed primarily to thermodynamically stabilization, rather than to kinetic stabilization associated with non-cooperative unfolding, evidently such a remarkable rate-dependence of unfolding on the temperature of incubation would not have been observed over such a large range of temperature.

The second important observation is that unfolding occurs so slowly at most of the temperatures at which monitoring was done. Classically, proteins that are purely thermodynamically stabilized achieve conformational equilibrium upon incubation at any temperature within a timescale of less than 300 s. In the present case, changes continue to be seen for over 4 h upon incubation of MbuTIM at 60 °C.

The third important observation is that MutMbuTIM unfolds significantly more slowly than MbuTIM at all temperatures examined, except for 83 °C (Fig. 5A to F). This clearly establishes that the kinetic thermal stability of MbuTIM is profoundly altered (i.e., the protein's unfolding is profoundly slowed down) by the introduction of just three ion pair interactions involving 4 mutations in a short stretch of residues located in one of the protein's eight helices (which is a part of its fourth beta\alpha unit).

3.5. Both MbuTIM and MutMbuTIM have two semi-independently-unfolding subdomains

We subjected MbuTIM and MutMbuTIM to differential scanning calorimetry (DSC) with heating carried out at a rate of 1 °C/min. Supplementary Figs. 8 and 9, respectively, show the DSC data for the two proteins, collected on a TA-Waters micro-DSC instrument. The deconvoluted data reveals the occurrence of two separate transitions, in both proteins. In MbuTIM, the transitions occur at 71 °C and 74 °C, combining to give an apparent transition at 73 °C which was also observed in the CD data. In MutMbuTIM, the two transitions occur at 78 °C and 80 °C, combining to give an apparent transition at 79 °C, which is close to the transition also seen in the CD data.

The observation of two distinct unfolding transitions in the DSC studies points to the presence of two independently-unfolding subdomains. During the last decade, studies with TIM-barrel structures of proteins like HisF have revealed that TIM barrels could have potentially evolved through the duplication of two ancestral half-barrel sub-domains [38,39]. In all likelihood, therefore, these early DSC studies point to the unfolding of two independent half-barrel subdomains in MbuTIM comprising 4 beta\alpha units each, from the eight beta\alpha units of MbuTIM's barrel structure.

We decided to follow these DSC studies up with more sensitive DSC measurements using the VP-DSC instrument from MicroCal, performing temperature scan rate-dependence studies involving very careful reference measurements made with buffers and with cells being allowed to stabilize their thermal history extensively over multiple scans using blanks (buffers lacking protein). Fig. 6A shows the results obtained with MbuTIM. It is clearly seen that there are two dominant DSC transitions, as noted earlier, but that the best fits of data can be obtained only by fitting the enthalpic envelope to three or four transitions, rather than just two transitions (although fitting to two transitions provides a reasonable fit of the main transitions). Interestingly, a profound difference in the shape of the unfolding enthalpic envelope can be seen with variations in scan rate. Fig. 6A establishes that as there is a progressive reduction in scan rate, transitions occur at progressively lower temperatures in MbuTIM. The relationships of scan rate with transition temperatures (Tm values) for four transitions followed over a wide range of scan

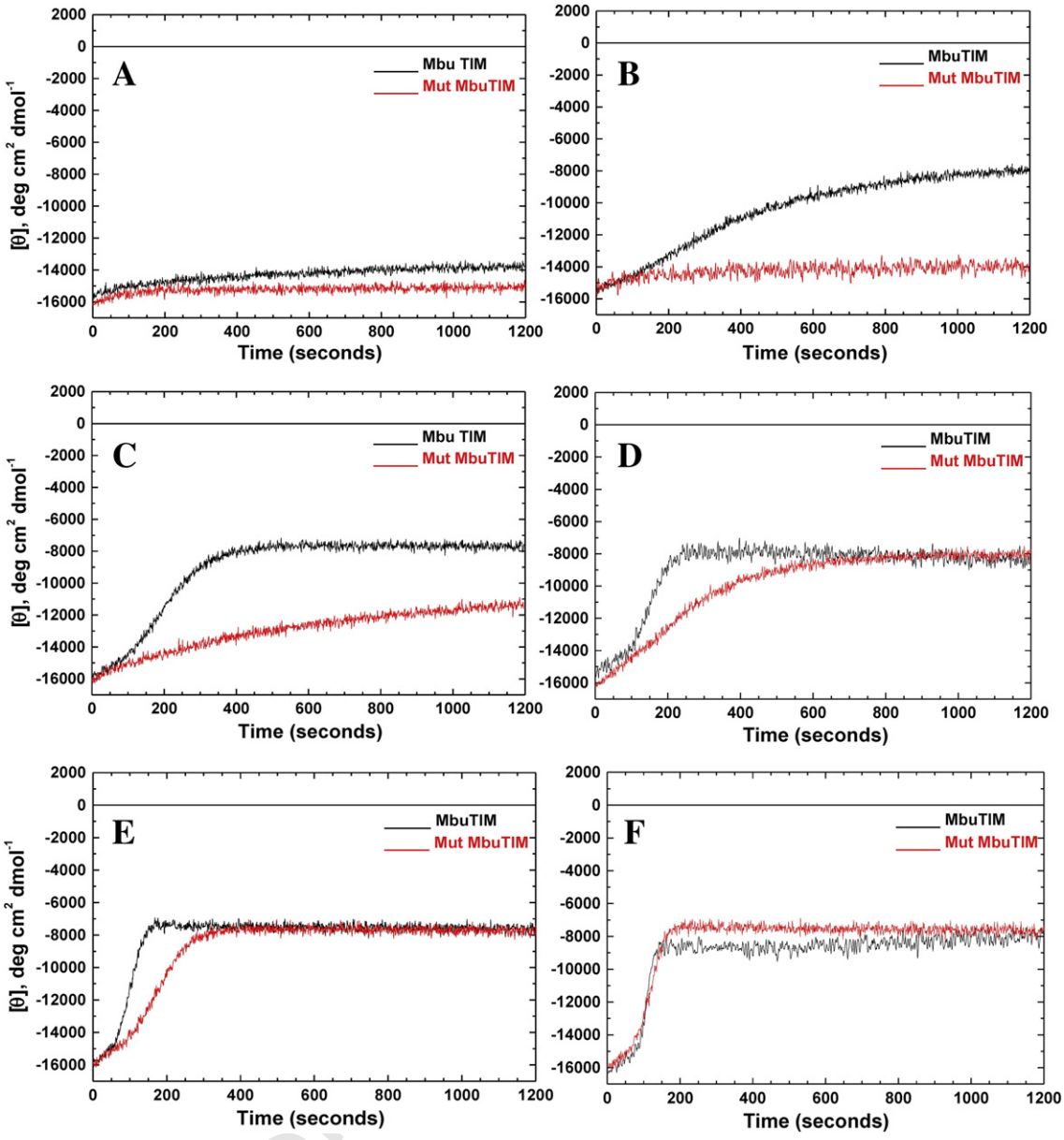

Fig. 5. Comparison of kinetics of heating-induced structural loss. Loss of secondary structural content for MbuTIM (black) and MutMbuTIM (red), monitored through changes in mean residue ellipticity at 218 nm over an incubation period of 20 min, as a function of temperature, with data shown for temperatures of 60 °C (panel A), 69 °C (panel B), 73 °C (panel C), 76 °C (panel D), 79 °C (panel E), and 83 °C (panel F).

rates is tabulated in Table 1. In Fig. 6A, it is also seen that certain very-broad transitions are spread out over a large range of temperatures at the lower scan rates of 15, 30 or 60 °C/h, and that these broad transition gain prominence at these scan rates. Fig. 6B provides comparative data, allowing a comparison between the 90 °C/h DSC scans for MbuTIM (Fig. 6A; bottom panel) and MutMbuTIM (Fig. 6B; middle panel). In this comparison, the difference seen between MbuTIM and MutMbuTIM in our earlier DSC studies on the TA-Waters instrument are validated, in that the two transitions in MutMbuTIM both occur at higher temperatures than the corresponding transitions in MbuTIM, although the details of the temperatures and scan rates involved, and the sensitivities of measurement, are different. Another interesting feature is the profound change effected in the DSC scans of both MbuTIM (Fig. 6B; top panel) and MutMbuTIM (Fig. 6B; bottom panel) upon addition of salt. The addition of salt profoundly increases the transition temperatures, instead of effecting a lowering, and this is seen in both MbuTIM and MutMbuTIM (see also Table 1). This indicates that the elimination, or attenuation, of electrostatic interactions (not just in the 4th beta-alpha unit, but in the proteins as a whole) results in stark compensatory increases in the strengths of other forces holding the structures of these proteins together (e.g., hydrophobic interactions and hydrogen bonding), resulting in very remarkable increases in transition temperatures. In the case of MutMbuTIM, whereas the introduction of the ion pairs resulted in increases of only 4–6 °C in transition temperatures, the presence of 2 M NaCl resulted in nearly a further 10 °C rise in transition temperatures as well as a collapse of the multiple transitions into two transitions. Salt thus appears to prevent at least some of the autonomous minor unfolding transitions, restricting the transitions to the two predominant (presumed) half-barrel unfolding transitions, with enhanced stability to unfolding.

It may be noted that in the work presented here, we decided to introduce into MbuTIM only three out of the four ion pair interactions that are engaged in by the corresponding helix of the $(\beta/\alpha)_4$ of PfuTIM. The reason was that the fourth ion pair interaction (which we did not initially introduce) involves only one residue from the 4th beta/alpha unit of PfuTIM and the second residue from the 3rd

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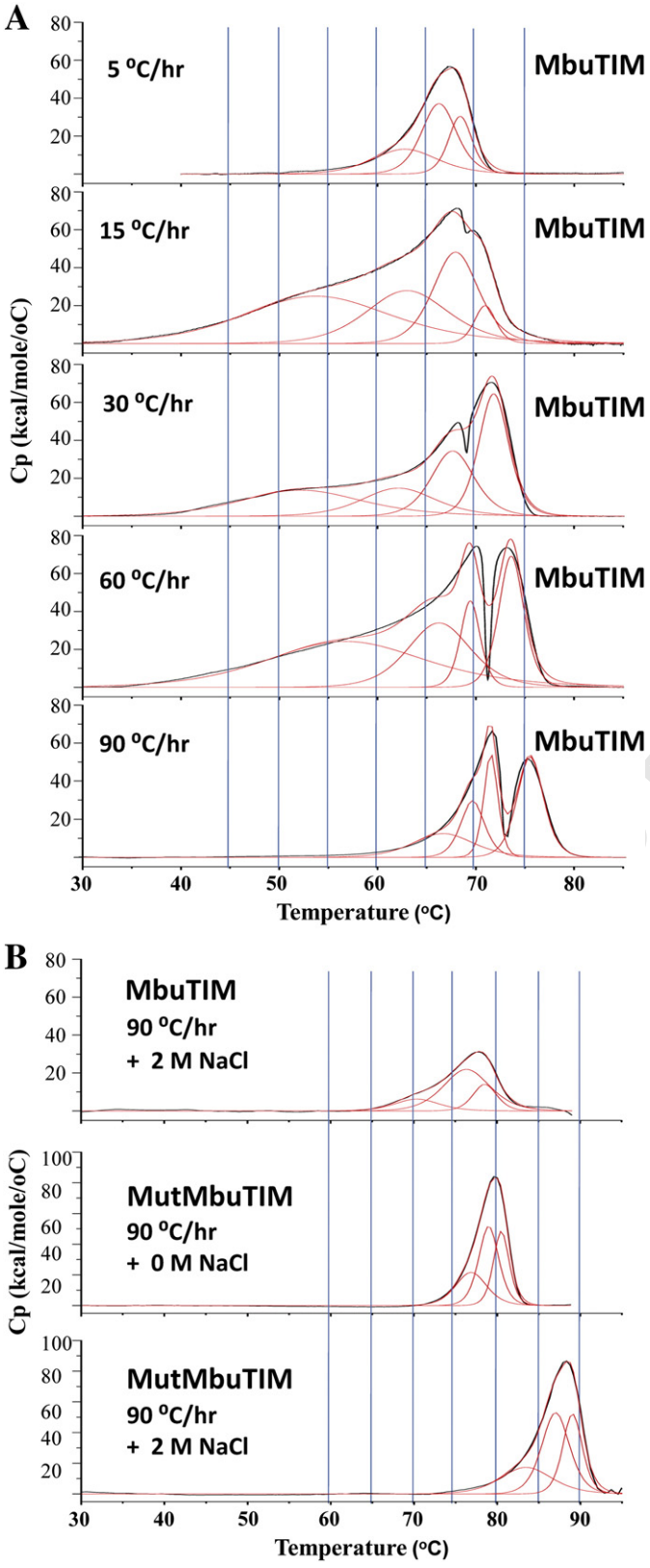

Fig. 6. DSC data for the endothermic structure-melting transitions. Panel A: MbuTIM subjected to thermal unfolding at different scan rates. Panel B: MbuTIM and MutMbuTIM subjected to thermal unfolding at comparable scan rates, with scans exploring the effects of the presence of salt (2 M NaCl). The thermograms are shown with fits of up to four individual transitions, after baseline flattening and deconvolution.

beta/alpha unit of PfuTIM which is placed at a considerably distal location. In the present study, we wished to restrict ourselves entirely to the 4th beta/alpha unit, since we wished to examine the consequence of stabilizing a single sub-domain element of structure upon the behavior of the entire MbuTIM. In passing, however, we would like to mention that in a separate set of experiments that we do not provide details for here, we also introduced the 4th ion pair interaction to create a further variant of MutMbuTIM, which we called MutMbuTIM-2. However, there was no significant difference between the behaviors of MutMbuTIM, containing 3 out of PfuTIM's 4 ion pairs, and MutMbuTIM-2, containing all 4 ion pairs. The unfolding data obtained for MutMbuTIM-2 was virtually identical to that obtained for MutMbuTIM.

4. Conclusions and perspectives

We had begun this work with the objective of examining whether it is possible to turn a psychophile protein of poor intrinsic thermal stability into a protein of significantly higher kinetic (and possibly also, higher thermodynamic) stability. To our surprise, we found that MbuTIM is a protein of considerable intrinsic thermal stability. One potential reason for this could be the fact that the protein is multimeric, since such proteins often turn out to be more stable than monomeric proteins. However, our experience with studying many different proteins suggests that it is unlikely that the entire explanation for the protein's high stability lies in its being multimeric. Despite the finding of surprisingly high stability in a psychrophile-derived protein, we went ahead with our objective of introducing a network of ion pair interactions within the surface of just one helix located in MbuTIM's 4th beta\alpha unit to see whether this enhances the protein's kinetic thermal stability. The idea was to compare the effects of these changes with the effects of the exactly reciprocal (opposite) residue changes introduced in the analogous region of a hyperthermophile protein, PfuTIM, i.e., to see whether the introduction of PfuTIM's ion pairs into the 4th beta\alpha unit increase the kinetic stability of MbuTIM, just as the introduction of MbuTIM's analogous sequence (in the region of the ion pairs) resulted in a decrease in the kinetic stability of PfuTIM, as already demonstrated [25].

The results reported here indicate that, indeed, the introduction of the ion pair network profoundly affects the behavior of two semi-independently-unfolding subdomains in MbuTIM, enhancing the temperatures at which both display unfolding in DSC experiments and also significantly slowing down the unfolding of MbuTIM, in the bargain. Our results suggest that the introduction of the ion pairs is affecting the

Table 1
Tm values of unfolding transitions in MbuTIM and MutMbuTIM at different scan rates and in the presence and absence of salt.

Protein scan rate	Peak 1 T_m (°C)	Peak 2 T_m (°C)	Peak 3 T_m (°C)	Peak 4 T_m (°C)
MbuTIM 5 °C/h	68.41	66.26	62.81	
MbuTIM 15 °C/h	70.94	67.96	63.08	54.04
MbuTIM 30 °C/h	71.83	67.68	62.20	52.37
MbuTIM 60 °C/h	73.62	69.45	66.36	57.06
MbuTIM 90 °C/h	75.55	71.54	69.68	66.73
MbuTIM 90 °C/h +2 M NaCl	78.55	76.38	70.4	
MutMbuTIM 90 °C/h	80.51	79	76.89	
MutMbuTIM 90 °C/h +2 M NaCl	89.06	87.03	83.5	

cooperativity of the unfolding process at two levels. Firstly, the stabilization of the 4th beta\alpha unit may be increasing its structural autonomy and causing an increase in the kinetic stability of the first half-barrel involving the first four beta\alpha units of MbuTIM. Secondly, this increase in the structural autonomy of the first half-barrel appears to be increasing the kinetic stability of the whole protein, i.e., the slower unfolding (and greater degree of survival) of the first half-barrel at high temperatures affords the second half-barrel the chance to remain packed against the first half-barrel to a greater degree, in turn effecting a slowing down of its unfolding, and an increase in its apparent temperature of structure-melting. Of course, the alternative (trivial) explanation remains that we have simply stabilized a transition-state (or states) relating to the unfolding of one or both presumed sub-domains, resulting in a raising of the activation barrier relative to the ground state, leading in turn to an increase in kinetic stability. In other words, the mutations we've made could be present in that part of the protein which remains folded in the transition state of the mutant, but which unfolds in the transition state of the wild type MbuTIM.

To the best of our knowledge, this may be one of the earliest instances in which it has been specifically demonstrated that the deliberate enhancements of certain non-covalent interactions (in this case, ionic interactions) within a single sub-domain element of structure in a protein domain, or motif, can result in a slowing down of the unfolding of the entire protein. This tentatively supports the hypothesis that a slowing down of unfolding rates (i.e., an enhancement of kinetic stability) can be deterministically elicited by deliberately reducing the cooperativity with which global unfolding tends to occur in a protein subjected to some structure-perturbing influence (in this case, heat), through enhancement of the stability of small sub-domain elements of structure. The reason this works is because such 'stabilized' sub-domain elements of structure then become independent of the requirement for packing interactions with other, neighboring elements of structure, and no longer cooperate with them during unfolding. Together with our previous demonstration that the removal of the ion pair network in the same sub-domain element of structure causes a hyperthermostable TIM protein to become much more of an 'ordinarily' thermostable protein, with greatly reduced kinetic stability [25], we think that a strong case has been made for how surface ionic interactions aid in determining protein kinetic stability as well as how these may be specifically engineered to manipulate kinetic stability.

There are two other pieces of work that predate some of our work to which we would like to draw special attention; one of these discusses inter-species variations in the kinetic stabilities of TIM proteins in the light of solvation-barrier free energies [40], while the other is a general treatment of structure-based design of kinetic stabilities in proteins [41]. While neither of these specifically draws attention to kinetic stability arising from non-cooperativity amongst protein sub-domains, we think that our approach and perspectives might someday blend with the perspectives presented in these papers to simultaneously derive both a thermodynamic and energetic explanation and a mechanical (rather than mechanistic) explanation for kinetic stability.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbapap.2013.01.001.

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