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Slow irreversible unfolding of *Pyrococcus furiosus* triosephosphate isomerase: Separation and quantitation of conformers through a novel electrophoretic approach

Sourav Mukherjee, Swati Sharma, Sanjeev Kumar, Purnananda Guptasarma *

Institute of Microbial Technology, Chandigarh 160 036, India

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Abstract

The thermostability of hyperthermophile proteins is not easily studied because such proteins tend to be extremely recalcitrant to unfolding. Weeks of exposure to structurally destabilizing conditions are generally required to elicit any evidence of conformational change(s). The main reason for this extreme kinetic stability would appear to be the dominance of local unfolding transitions that occur within different parts of the structures of these molecules; put differently, local sub structural unfolding transitions that occur autonomously and reversibly are thought to fail to cooperate to bring about global unfolding in a facile manner, leading to a low overall observed rate of unfolding. For reasons that are not yet fully understood, unfolding is also reported to occur irreversibly in hyperthermophile proteins. Therefore, conventional experimental approaches are often unsuited to the study of their unfolding. Here, we describe a novel electrophoretic approach that facilitates separation, direct visualization, and quantitation of the folded, partially folded, and unfolded forms of the hyperthermophile protein triosephosphate isomerase from *Pyrococcus furiosus*, produced in the course of its irreversible structural destabilization by the combined action of heat and chemical agents. Our approach exploits (i) the irreversibility of global unfolding effected by heat and denaturants such as urea or guanidine hydrochloride, (ii) the stability of the native form of the protein to unfolding by the anionic detergent sodium dodecyl sulfate, (iii) the differential susceptibilities of various protein conformations to being bound by SDS, and (iv) the differential electrophoretic migration behavior displayed as a consequence of differential SDS binding.

Keywords: Hyperthermophiles; Protein folding; kinetic stability; Electrophoretic separation; Slow unfolding

The measurement of parameters associated with protein thermodynamic stability requires that protein unfolding be fully reversible. Proteins from hyperthermophiles, however, almost always unfold irreversibly [1–4]. Conventional methods of examining thermodynamic stability cannot be straightaway applied, therefore, to the study of these proteins [5,6]. In recent years, however, a novel spectroscopic method based on measurement of rates of exchange of amide protons during reversible cycling of molecules between different conformations has been used successfully to examine hyperthermophile protein stability, without attempting to effect global unfolding [7–9]. This new method utilizes the observation [10] that minor, localized unfolding transitions occur continually within such proteins even under native conditions. Such rigorous measurements of stability, made with homologous hyperthermophile and mesophile proteins at their respective temperature optima of function, have lent support to a growing view that hyperthermophile proteins are only marginally more stable than their mesophile counterparts. To appreciate this point in somewhat greater detail, let us consider what is known about, e.g., the thermodynamic stability of a *Pyrococcus furiosus* (pfu)-derived hyperthermophile homolog of the protein rubredoxin, which consists of a single polypeptide, 53 amino acids long.

* Corresponding author. Fax: +91 172 2690585.
E-mail address: pg@imtech.res.in (P. Guptasarma).

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The change in free energy associated with the unfolding of *P. furiosus* rubredoxin at 100 °C (ΔG_{unf}) has been measured to be only about 15 kcal/mol [3]. It may be noted that this is a very ordinary measure of ΔG_{unf} for any protein, let alone one from a hyperthermophilic organism. As is well known, 15 kcal/mol falls within the range of ΔG_{unf} expected to be associated with the unfolding of mesophile proteins (5–15 kcal/mol), and this value is equivalent to the energy required to break only a few hydrogen bonds per protein molecule [11]. Again, similarly low estimates of ΔG_{unf} have been reported for a number of other hyperthermophile proteins [12–14], raising the question of how such manage to retain their structure and function at high temperatures without being excessively thermodynamically stabilized.

It emerges that the answer probably lies in the kinetics of the unfolding of these proteins rather than in the thermodynamics associated with unfolding. Studies of unfolding rates (rather than of free energies associated with unfolding) have now begun to reveal that much of the apparent stability of hyperthermophile proteins could be due to kinetic factors. Hyperthermophile proteins display extreme kinetic stability [15–17]. For example, rubredoxin from *P. furiosus*, which is thermodynamically stabilized by only 15 kcal/mol, unfolds at a rate of $10^{-6}\,s^{-1}$ at 100 °C, making it the slowest unfolding protein known to man [10]. Similar reports of extraordinarily slow unfolding have also emerged from studies of other hyperthermophile proteins [18–20], indicating that these proteins display extreme kinetic stability. [It may be noted that the use of the word 'stability' here in relation to the slowness of unfolding of a protein invokes only the general meaning of this word as it is used in the English language. Technically, the word 'stability' cannot really be separated from considerations of the energies of stabilization of a system or a molecule; yet, the term "kinetic stability" has already entered the literature.] From the point of view of protein functionality alone, it may be appreciated that kinetic stability can be at least as important a determinant of functionality as thermodynamic stability in harsh physical/chemical protein environments. In principle, a population of a slowly unfolding protein that is placed under thermodynamically destabilizing conditions (e.g., a temperature higher than the equilibrium melting temperature) but that takes weeks or months to attain unfolding equilibrium could potentially continue to exist and display function over such long periods of time. Such a population could potentially even outperform a homologous protein that is more thermodynamically stable but that undergoes unfolding at a faster rate when placed under thermodynamically destabilizing conditions. At least in theory, therefore, protein engineering efforts in future years might consider focusing on attempts to slow the unfolding as an additional means of achieving longer survival of structure and function in harsh environments.

Clearly, given the above facets of hyperthermophile protein stability, any simple structural–biochemical method allowing researchers to follow irreversible unfolding as a rough measure of kinetic stability could be of great value. In fulfillment of such a need, we describe here a novel method that employs SDS–PAGE gel electrophoresis, with certain essential modifications, to examine kinetic stability. We have tested this method with the recombinant form of triosephosphate isomerase (TIM)[1] encoded by the genome of *P. furiosus*, produced through heterologous overexpression in *Escherichia coli*. The rationale of the approach is described below in some detail.

The electrophoretic mobility of a protein on SDS–PAGE can be directly correlated with its molecular mass. However, this relationship holds only if the protein happens to fully unfold and bind SDS to saturation prior to its exposure to an electric field. If unfolding occurs to an incomplete extent for any reason, a protein could be expected to display a mobility that is different from that ordinarily expected. This could occur in two conceivable ways: (1) a lower mobility than expected resulting from a poorer-than-normal level of binding of SDS (due to nonexposure of hydrophobic residues). Poor binding of SDS could be anticipated to result in a lower net level of negative charge available on the protein during electrophoresis. or (2) a higher mobility than expected resulting from the compactness of conformation due to incomplete unfolding because such a conformation would generally be associated with a smaller-than-normal hydrodynamic volume of the molecule, facilitating greater mobility in the gel matrix due to a lesser overall retardation of the protein's movement. Precisely which of these influences would actually determine the mobility of an incompletely unfolded protein would, of course, be expected to depend on the degree of survival of structure. Therefore, if a "sufficient" number of SDS molecules (in keeping with the protein's size) were to bind to a protein, a greater mobility than expected would be observed because of the compactness of conformation. On the other hand, despite such compactness of conformation, if there were "insufficient" binding of SDS, a lesser mobility than anticipated could result due to the relative insufficiency of negative charge associated with the protein molecule. Of course, such anomalies of mobility are not ordinarily observed. This is because preparation of protein samples for SDS–PAGE normally involves the use of a combination of two destabilizing influences to ensure complete protein unfolding and binding of SDS to saturation. These are (i) the presence of chemical agents to denature and reduce the protein (e.g., SDS and dithiothreitol/β-mercaptoethanol) and (ii) the heating of the protein in the presence of the above agents, through boiling of the sample for a few minutes, to aid in its denaturation.

With most mesophile-derived proteins, elimination of the heating step does not affect the mobility seen (see, e.g., Fig. 1B). This is probably because mesophile proteins, in any case, tend to be easily and completely unfolded by the presence of a combination of SDS and a reducing agent, without requiring the additional step of boiling in the pres-

[1] *Abbreviations used:* TIM, triosephosphate isomerase; IPTG, isopropyl β-D-thiogalactoside; SLB, SDS–PAGE loading buffer.

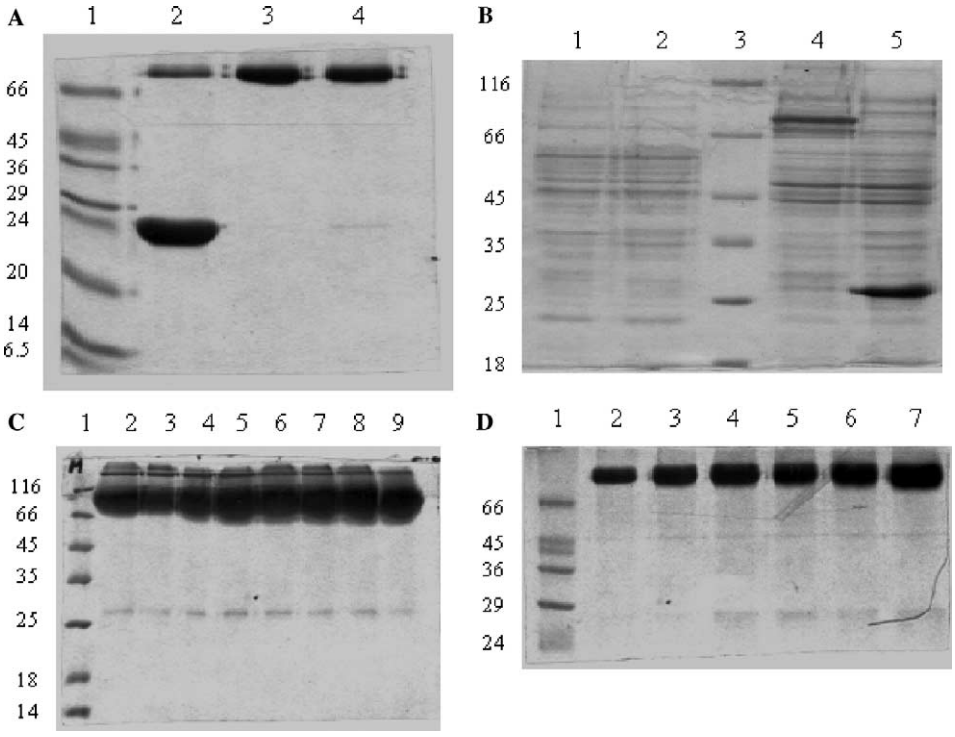

Fig. 1. Separating folded and unfolded forms of Pfu TIM. (A) Purified Pfu TIM added to SLB and boiled (lane 2), added to SLB but not boiled (lane 3), and boiled, cooled, added to SLB, and electrophoresed without further boiling (lane 4). (B) Lysates of *E. coli* M-15 cells in the presence of SLB, electrophoresed without sample boiling (lane 1) and with sample boiling (lane 2). Lysates of *E. coli* M-15 cells expressing Pfu TIM in the presence of SLB, electrophoresed without sample boiling (lane 4), and with sample boiling (lane 5). (C) Purified Pfu TIM incubated in the presence of SLB for 1, 3, 4, 5, 7, 8, 9, and 10 days, respectively (lanes 2–9), and electrophoresed without sample boiling. (D) Purified Pfu TIM incubated at 98 °C for 5 min, 2, 5, 10, 15, and 20 h, respectively (lanes 2–7) and then cooled, added to SLB and electrophoresed without sample boiling. In all panels, mobilities corresponding to different molecular weights are marked.

ence of SDS for unfolding to occur to completion. In contrast, with a hyperthermophile protein, it is possible that the heating step would be essential, i.e., SDS and the reducing agent could, by themselves, be unable to effect unfolding when samples are not also boiled in the presence of these chemicals. A gel-based unfolding assay exploiting this observation could use two other useful features/aspects of the behavior of hyperthermophile protein molecules: (i) their irreversible unfolding behavior, which would allow molecules to remain essentially unfolded after unfolding, and (ii) the differential use of heat in the experiment, sometimes to attempt thermal unfolding prior to electrophoretic evaluation performed without boiling of samples in the presence of SDS and sometimes to unfold samples during preparation for electrophoresis through boiling in the presence of SDS.

Thus, for experiments in which unfolding of *P. furiosus* TIM would be achieved (prior to electrophoretic evaluation) through heat-induced and/or chemical-induced unfolding, it was determined that elimination of the boiling step during sample preparation for electrophoresis could potentially facilitate a separation of the "still-folded" protein populations from the "irreversibly unfolded" populations, since molecules failing to unfold prior to electrophoresis would be expected to fail to bind SDS sufficiently in the absence of the boiling step and thus migrate differently. Such separation could allow (i) folded and (ii) irreversibly unfolded populations created before electrophoresis to be separated and quantitated later. As described below, our results show that the concepts espoused here are largely tenable.

Materials and methods

Proteins

Pyrococcus furiosus genomic DNA was obtained as a gift from Dr. M.W.W. Adams (University of Georgia, Athens, GA, USA). The gene encoding Pfu TIM was PCR amplified from this genomic DNA using suitable forward (5′-ATATTAATAGGATCCGCTAAACTCAAG-3′) and reverse (5′-ATATTAATAAAGCTTCTACTCCTTAAT-3′) primers. The PCR product was digested with *Bam*HI and *Hin*dIII and cloned into the multiple cloning site of the vector pQE-30 (Qiagen, Germany) for expression in fusion with an N-terminal 6×His affinity tag. The sequenced clone was transformed into *Escherichia coli* M-15 (Qiagen), and the protein was expressed through induction with IPTG after approximately 3 h of growth of cells at 37 °C ($A_{600} \sim 0.6$–0.8). Cells were harvested after growth for a further 4 h of incubation in the presence of IPTG, lysed in the presence of 8 M urea, and purified through chromatogra-

phy on a Ni-NTA affinity column (Qiagen) as per standard protocols provided by the manufacturer. Urea was dialyzed out following purification, and the soluble protein was stored in 20 mM Tris, pH 10. Separately, protein was also purified in the absence of urea by an alternative standard protocol provided by the manufacturer and found to be soluble and otherwise identical to the protein purified in the presence of urea in all respects. For all experiments described, protein stocks were diluted in a ratio of 1:9 with 50 mM phosphate buffer, pH 7.3, containing 300 mM KCl, to give rise to a protein concentration of approximately 1.4 mg/ml.

Electrophoresis

SDS–PAGE electrophoresis was carried out using gels of acrylamide percentages varying between 10 and 15% for the resolving gel and 5% for the stacking gel, with standard gel compositions and protocols, however, there was one exception. Samples were not always boiled after the addition of the SDS–PAGE loading buffer (SLB) containing β-mercaptoethanol and SDS. Such treatment was based on the following observations. Boiling of the sample in the presence of SLB effected denaturation of the whole population, yielding migration of the protein as a band of 25 kDa molecular weight, as would be expected for a monomeric polypeptide fully unfolded by SDS and heat. In contrast, failure to boil samples was seen to result in migration of the protein as a band of very poor mobility corresponding to about 80 kDa. This was because unfolding-coupled binding of SDS by Pfu TIM required application of both SDS and heat (boiling). Also, Pfu TIM was established to unfold irreversibly upon destabilization by any combination of heat and chemical denaturing agent. Thus, any population of protein molecules unfolded by other means prior to electrophoresis was observed to remain in a nonnative conformation until the addition of the SLB. Upon addition of SLB, such populations readily bound SDS and behaved like fully unfolded monomers saturated with SDS, except in a few cases where a gradation of binding behavior was seen. This allowed physical separation of conformationally distinct forms in a very facile manner.

Circular dichroism

CD spectroscopy was carried out using a Jasco J-710 spectropolarimeter fitted with a Peltier arrangement for regulating temperature, using cuvettes of path length varying from 0.1 to 0.5 cm. Mean residue ellipticities were determined and plotted both for spectra obtained at specific temperatures and for raw ellipticities measured under regulated conditions of heating, as a function of varying temperature. Protein concentrations of samples were determined using an extinction coefficient of 9770 at 280 nm, translating into an absorbance of 0.38 AU for 1 mg/ml.

Gel filtration chromatography

A Pharmacia SMART workstation fitted with a Superdex-200 microcolumn (bed volume 2.4 ml) was equilibrated either with phosphate buffer (50 mM, pH 7.3) or with the SDS-containing Laemmli (tank) buffer normally used for SDS–PAGE experiments. For samples run on the column equilibrated with SDS–PAGE tank buffer, samples were first previously equilibrated with SLB (lacking bromophenol blue). Such chromatography helped examine the hydrodynamic volumes of Pfu TIM samples under conditions both different from and similar to those used for gel electrophoresis to enable comparisons to be drawn.

Results and discussion

Distinguishing between folded and unfolded Pfu TIM

Fig. 1A (lane 2) shows that Pfu TIM prepared for SDS–PAGE analysis through boiling of the sample after addition of the SLB displays the exact mobility expected of a protein of 25.4 kDa. However, when SLB is added but samples are not subjected to boiling (lane 3), Pfu TIM displays a much lower mobility (due presumably to the inability of SDS alone to effect unfolding and binding when heating is not also done through boiling of the sample in the presence of SLB). The same poor mobility is also shown by samples that have been heated at 100 °C for 5 min and then cooled to room temperature, added to SLB, and electrophoresed without any boiling being done in the presence of SLB (lane 4).

This shows that quantitative unfolding and binding of SDS are seen only when samples are boiled in the presence of SLB, with the combination of heat and SDS effecting unfolding (lane 2); however, even under these conditions, a fraction of the population in lane 2 can be seen to have failed to unfold, migrating as a faint band of mobility corresponding to the bands seen in lanes 3 and 4 and indicating that the standard conditions of boiling with SLB are not necessarily sufficient to effect complete unfolding.

Our interpretation of the above observations is as follows. Only the presence of SDS alone in the form of the SLB (lane 3) or simple heating alone in the absence of the SLB (lane 4) cannot elicit unfolding and binding of SDS by Pfu TIM. Rather, a combination of heat (boiling for 5 min) and SDS (present in the SLB) effects complete unfolding and binding of SDS in a majority of the protein population (lane 2); however, even under these conditions, a fraction of the population fails to be unfolded and continues to be seen as a faint band of poor mobility near the top of the gel. This faint band corresponds exactly in mobility to that of the sample in lane 3, which is presumed to have remained structured (having failed to unfold in the presence of SDS alone, since samples were not boiled after addition of SLB). An identical situation is obtained with the sample in lane 4, which was also not boiled after addition of SLB. Even though this sample was previously boiled for 5 min prior to

the addition of SLB, such boiling clearly did not effect noticeable unfolding because proteins from *P. furiosus* are, in any case, not expected to be unfolded by exposure to a temperature of 100 °C, as this is very close to the optimal growth temperature of the organism and less than the organism's maximum survival temperature [21].

Fig. 1B verifies the results shown in Fig. 1A in the specific context of the cytoplasm of *E. coli* (strain M15) within which the Pfu TIM was produced through heterologous overexpression. Lanes 4 and 5 in the figure show that Pfu TIM is the only detectable protein in the cytoplasm of the transformed *E. coli* strain that displays different mobilities depending on whether samples are boiled or not boiled after addition of SLB. Lanes 1 and 2 in the figure serve as controls consisting of untransformed cells that demonstrate that no other protein in the cytoplasm shows this behavior. This is not surprising, since the proteins of *E. coli* (a mesophilic organism) are not expected to be hyperthermostable, whereas Pfu TIM is expected to be hyperthermostable.

Fig. 1C shows the results of a control experiment in which samples were incubated in the presence of SLB for up to 10 days at 25 °C to determine whether the failure of SDS to unfold Pfu TIM prior to electrophoresis is a kinetic effect due simply to an insufficient duration of incubation with SDS. The survival of the anomalous mobility over this entire period of days establishes that it is not and that SDS in the SLB alone is unable to effect unfolding.

Similarly, Fig. 1D shows the results of another control experiment in which samples were first incubated at 98 °C for up to 20 h, and then cooled, added to SLB, and electrophoresed on SDS–PAGE. These samples too were found not to have unfolded irreversibly to become susceptible to adequate SDS binding, suggesting that the result inferred from lane 4 of Fig. 1A above (concerning the failure of heat alone to unfold Pfu TIM) is also not a simple kinetic effect. Thus heating at a temperature close to 100 °C does not cause unfolding of the protein.

Unfolding Pfu TIM by a combination of heat and urea

Fig. 2A shows that heating of Pfu TIM at 98 °C in the presence of 50 mM urea effects a gradual change in the protein's mobility with time, due presumably to a progressive irreversible "unfolding" of small substructures allowing a greater level of binding of SDS when SLB is subsequently added. As the lanes in the figure show, over a period of 3 h of incubation, the mobility of Pfu TIM changes from an apparent molecular weight greater than 66 kDa to a much lower apparent molecular weight of about 45 kDa, with the faint appearance of a ~25-kDa monomer. However, as explained below in a section dealing with chromatographic evaluation of these samples under buffer conditions identical to those used for electrophoresis, the observed differences in mobility can be established to be due not to any changes in polypeptide molecular weight or in quaternary structural status per se but rather to partial unfolding into a conformation more susceptible to being bound by SDS.

Fig. 2B shows that incubation over the same 3 h at 98 °C in the presence of higher concentrations of urea (up to 500 mM) progressively elicits an apparently two-state transformation of significant fractions of the protein population from the species displaying a mobility corresponding to an apparent molecular weight of about 45 kDa to the completely unfolded monomer displaying a molecular weight of ~25 kDa. However, 500 mM urea fails to effect unfolding of the entire population. Fig. 2C shows that such

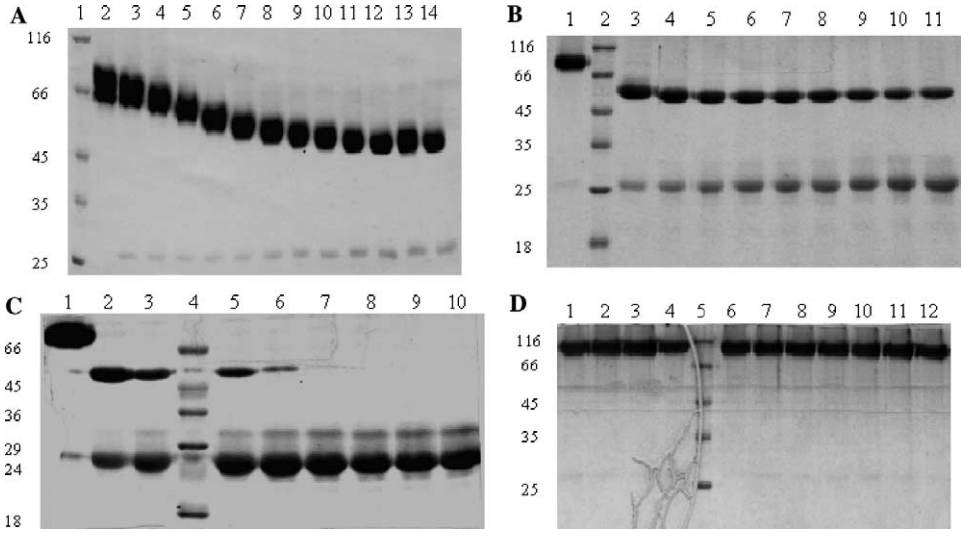

Fig. 2. Destabilization/unfolding of purified Pfu TIM by heat and urea. In all experiments, samples were electrophoresed after addition of SLB without sample boiling. (A) Pfu TIM incubated in the presence of 50 mM urea at 98 °C for 0, 15, 30, 45, 60, 75, 90, 105, 120, 135, 150, 165, and 180 min, respectively (lanes 2–14). (B) Pfu TIM incubated for 3 h at 98 °C in the presence of 0 (lane 1), and 50, 100, 150, 200, 250, 300, 350, 450, and 500 mM urea (lanes 3–11), respectively. (C) Pfu TIM incubated for 3 h at 98 °C in the presence of 0, 0.5, and 1 M (lanes 1–3), and 1.5, 2.0, 2.5, 3.0, 3.5, and 4.0 M urea (lanes 5–10), respectively. (D) Pfu TIM incubated for 12 h at 25 °C in the presence of 0.1, 0.2, 0.3, and 0.4 (lanes 1–4), 0.5, 0.6, 0.7, 0.8, 0.9, and 1.0 M urea (lanes 6–11), and 0 M urea (lane 12), respectively.

unfolding is achieved only by raising urea concentration further. Using incubations of 3 h and different concentrations of urea, it is observed that a urea concentration greater than 2 M (lane 6) is required to unfold the entire Pfu TIM population at 98 °C.

A control experiment (Fig. 2D) shows that incubation without concomitant heating for 12 h in the presence of urea alone (at 25 °C), using concentrations of urea ranging from 100 mM to 1 M, elicits no unfolding whatsoever. This demonstrates that the effects observed result from a combination of heating and urea.

Unfolding Pfu TIM by a combination of heat and GdnCl

No unfolding is observed upon incubation with GdnCl alone at 25 °C (data not shown). Even in the presence of 1 M GdnCl, Pfu TIM remains essentially folded for up to 20 h at 98 °C (Fig. 3A, lane 9). However, upon incubation at 98 °C with a higher GdnCl concentration of 2 M, significant unfolding is achieved over relatively short durations of incubation of only a few hours (Fig. 3B). Raising GdnCl concentration further to 4 M elicits unfolding of almost the entire population within 3 h of incubation at 98 °C (Fig. 3C). Unlike with urea, no partial structural changes are seen at low GdnCl concentrations, with the entire scheme of unfolding appearing to be two-state. Also, interestingly, lower concentrations of urea than GdnCl are required to effect complete unfolding over 3 h, even though GdnCl is the stronger of the two denaturants (given that it destabilizes electrostatic and hydrogen bonding interactions, unlike urea which destabilizes only hydrogen bonding interactions).

Pfu TIM is not unfolded by a combination of cold and urea/GdnCl

Since Pfu TIM is a hyperthermostable protein that has evolved to function in an organism with an optimal temperature of growth above 100 °C, we wished to use our electrophoretic assay to examine whether the protein might show any evidence of cold denaturation at low temperatures. However, no unfolding whatsoever was observed over days of incubation at 6 °C, either in the absence or in the presence of urea (4 M) and/or GdnCl (4 M), indicating that cooling and chaotropic agents do not combine to effect unfolding of this protein (data not shown).

Low urea concentrations do not alter primary or quaternary structural status

In Fig. 2A, there is a progressive change in mobility due to different durations of incubation with 50 mM urea at 98 °C. We know already that the polypeptide molecular weight of Pfu TIM is 25 kDa. Therefore, a question that remains inadequately addressed is whether the changes in mobility reflected in an apparent change in molecular weight down to 45 kDa in Fig. 2A are due to (a) changes in the primary structural status of Pfu TIM (e.g., involving cleavage of the protein at high temperature, in the presence of urea, to a lower-molecular-weight form that, however, remains multimeric and folded), (b) changes in the quaternary structural status of Pfu TIM (e.g., through dissociation of a higher-molecular-weight multisubunit form, such as a tetramer, into a lower-molecular-weight form, such as a dimer),

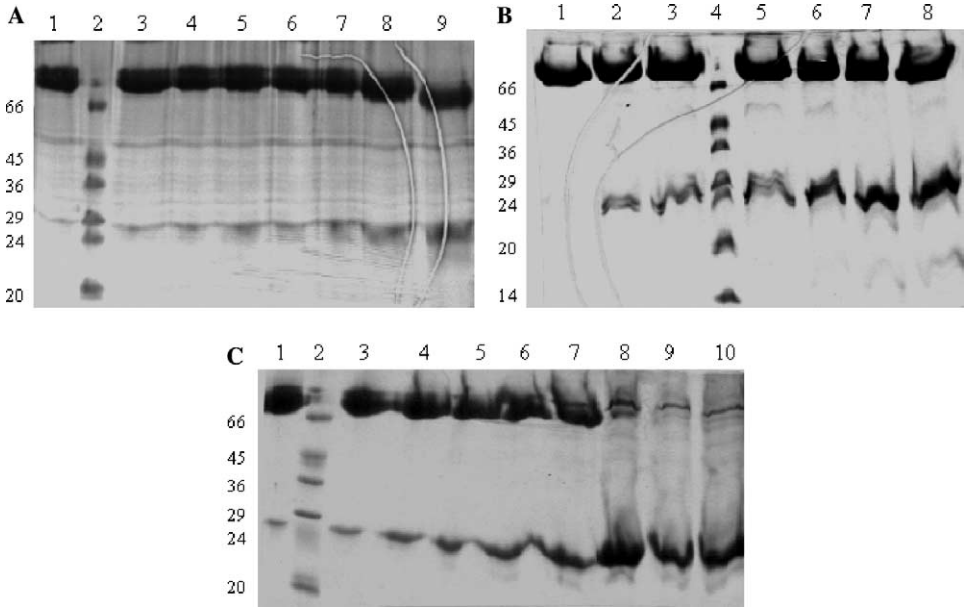

Fig. 3. Unfolding of purified Pfu TIM by heat and GdnCl. In all experiments, samples were electrophoresed after addition of SLB without sample boiling. (A) Pfu TIM incubated in the presence of 1 M GdnCl at 98 °C for 0 (lane 1) and 15, 30, 60, 120, 300, 600, and 1200 min, respectively (lanes 3–9). (B) Pfu TIM incubated in the presence of 2 M GdnCl at 98 °C for 0, 15, 30 (lanes 1–3) and 60, 120, 180, and 300 min, respectively (lanes 5–8). (C) Pfu TIM incubated for 3 h at 98 °C in the presence of 0 (lane 1) and 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, and 4.0 M GdnCl (lanes 3–10), respectively.

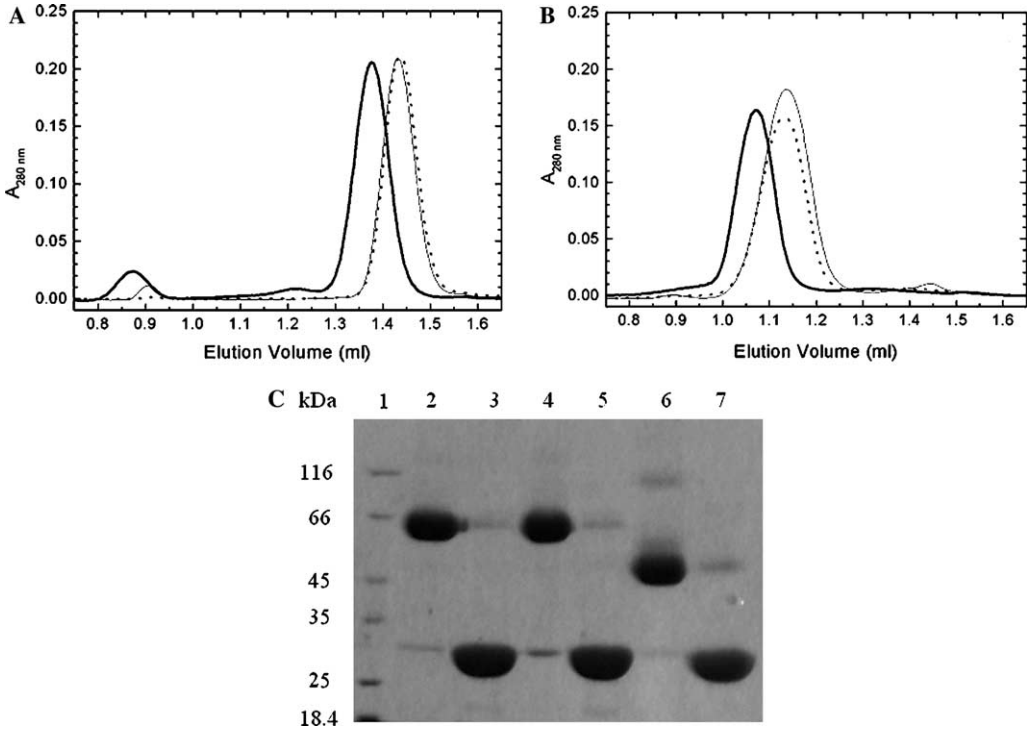

Fig. 4. Hydrodynamic volumes and electrophoretic behavior of samples bound differentially by SDS. (A and B) Elution chromatograms of Pfu TIM on a SMART Superdex-200 gel filtration column equilibrated and run either with 50 mM phosphate buffer of pH 7.3 (A) or with the tank buffer and sample preparation conditions (B) identical to those used for electrophoreses, however, without any boiling of samples after addition of SLB. (Note: no bromophenol blue was added, and no electric current was applied). The bold solid lines in A and B are profiles of samples heated for 3 h at 98 °C in 50 mM urea. The dotted lines are profiles of samples incubated for over 24 h at room temperature in 50 mM urea. The thin solid lines are profiles of Pfu TIM samples not incubated with urea. (C) Samples from A are shown electrophoresed on 10% SDS–PAGE, with boiling after SLB addition (lanes 3, 5, and 7) and without such boiling (lanes 2, 4, and 6), with lanes 2 and 3 corresponding to the elution shown with a thin solid line in (A), lanes 4 and 5 corresponding to the elution shown with a dotted line, in (A), and lanes 6 and 7 corresponding to the elution shown with a bold solid line in (A).

(c) changes in the conformational status of TIM (e.g., through partial unfolding of each subunit in the multimeric state, leading to greater SDS-binding potential and, therefore, to a greater mobility of the SDS-bound multimeric state in the gel during electrophoresis), or (d) effects that are artifacts introduced by the gel electrophoretic procedure. To address this, we performed the following experiments.

First we performed gel filtration chromatography using a Superdex-200 column equilibrated with phosphate buffer (50 mM, pH 7.3) to examine the effects on the protein of heating in the presence of urea; however, in this instance, exposure of the protein to SLB was avoided at all stages, and no attempt was made to replicate the gel electrophoretic environment during gel filtration. Fig. 4A shows that the elution profile of a protein sample never exposed to urea is identical to the elution profile of a protein sample incubated in 50 mM urea for over 24 h at room temperature prior to loading onto the column. Both of these elutions occur at the expected elution volume (1.43 ml) for a tetrameric state of Pfu TIM for this particular column. However, when the protein is heated at 98 °C for 3 h in the presence of 50 mM urea (similar to the treatment of the sample in lane 14 of Fig. 2A), cooled, and then loaded onto the column, it now elutes at an earlier volume of 1.34 ml. This suggests immediately that heating in the presence of urea results in an increase in hydrodynamic volume that could be due to partial unfolding of subunits in the multimeric, tetrameric state. Notably, if the protein had undergone dissociation, e.g., into dimers, the elution could have been expected to occur at a volume much later than 1.43 ml (at approximately 1.60 ml) rather than at the earlier elution volume of 1.34 ml which is seen, because dissociation would have reduced the protein's hydrodynamic volume. Therefore, dissociation can be ruled out as an explanation for the 45-kDa species in lane 14 of Fig. 2A.

Second we performed gel filtration on the same column with pre equilibration of the column being carried out with the tank buffer ordinarily used for gel electrophoresis, in place of the phosphate buffer used for the previous experiment just described. For these experiments, importantly, the protein sample was also first added to SLB (prepared without bromophenol blue) and second loaded onto the column, ensuring that the protein would remain in the presence of SDS at all times in a manner similar to that obtaining during gel electrophoresis (except, of course, for the fact that there would be no electric field acting on SDS-bound protein species during gel filtration). A further important point to note here is that the Superdex-200 resin is known to be mildly hydrophobic, for which reason salt (at a level of 100–150 mM) is often included during gel filtration to

prevent any affinity of proteins possessing hydrophobic surfaces for the column resin matrix. Given this, a saturating presence of SDS in the bulk solvent could be expected to result in a saturation binding of the hydrophobic tail of SDS to the hydrophobic surfaces on the resin over the entire column, converting it into a negatively charged column matrix that would now impact on the elution behavior of any negatively charged protein species (particularly, any SDS-bound species). Therefore, all SDS-bound protein species (ranging from very poorly bound to completely unfolded and saturation-bound species, regardless of their levels of SDS binding) could be expected to elute at volumes earlier than normal simply because the proteins (now each negatively charged by SDS to various levels) would be reluctant to enter into the SDS-coated and negatively charged void spaces within the resin beads due to charge–charge repulsions. Indeed, this is what we discovered to be happening.

Fig. 4B shows that, despite the presence of the SDS, the elution profiles of Pfu TIM incubated in 50 mM urea for over 24 h at room temperature remain identical to those of Pfu TIM not exposed to urea, however, with both samples eluting at a much earlier elution volume (1.13 ml) than the normally expected volume of 1.43 ml. Again, as observed previously (Fig. 4A) with runs that used equilibration with phosphate buffer rather than with gel electrophoresis tank buffer, Fig. 4B shows that protein sample heated in 50 mM urea at 98 °C for 3 h prior to cooling and loading displayed a decreased elution volume (1.07 ml) indicative of an increased hydrodynamic volume. Therefore, despite the apparent partial exclusion of SDS-bound protein forms from the interiors of the beads constituting the gel matrix—and the resulting earlier elutions of all species—what was consistently observed was that protein heated in the presence of urea (similar to lane 14 of Fig. 2A) was altered conformationally (i.e., partially unfolded) in a manner that was detectable both using regular gel filtration and using gel filtration attempting to simulate SDS–PAGE gel electrophoresis without the presence of an electric field. This shows that the splitting of the protein population into distinguishable conformers is not an artifact of the gel electrophoretic procedure or the specific environment obtaining during electrophoresis. Simultaneously, this also shows that the protein that has been heated and cooled remains unable to refold/transform to its fully native state, regardless of the method used to separate conformers. Therefore, it may be concluded that the gel environment neither creates any artifacts of separation nor influences the kinetics of interconversions of species; the protein that has been subtly altered through heating in the 50 mM urea for 3 h at 98 °C is truly different from the native form and remains unable to reconvert to the native form. Both gel filtration and gel electrophoresis manage to allow us to distinguish between the two conformers. Certainly, however, the separation effected by gel electrophoresis is much more dramatic owing to the differential SDS-binding potential translating into very different mobilities under an electric field.

We ran the three samples on the Superdex-200 column equilibrated with phosphate buffer (Fig. 4A) on a 10% SDS–PAGE (Fig. 4C), with addition of SLB to all samples, and with each sample being electrophoresed both with and without boiling in the presence of SLB. As Fig. 4C shows, the sample that was not exposed to urea displayed the expected difference in mobility resulting from boiling with SLB (lane 3) and failure to boil after addition of SLB (lane 2). Likewise, the sample incubated with 50 mM urea at room temperature also displayed the exact same mobility with the boiled sample (lane 5) as that seen earlier with the boiled sample not exposed to urea (lane 3), as was also the case with the sample that was not boiled after addition of SLB (lane 4) which showed the same mobility as that seen earlier in lane 2. Interestingly, however, whereas the sample heated with urea and boiled (lane 7) also showed the same mobility corresponding to a ~25-kDa band as seen with all boiled samples previously, this was not true of the sample that was added to SLB but not boiled (lane 6). The sample in lane 6 displayed an apparent mobility of a ~45-kDa band as seen earlier (lane 14, Fig. 2A), validating the results and interpretations of the gel filtration data presented earlier.

Importantly, the gel electrophoresis data in Fig. 4C demonstrate that all boiled samples have an apparent mobility of ~25-kDa, which rules out the possible generation of a ~45-kDa band through cleavage of polypeptide chains associated into a multimer. Therefore, the only explanation that remains unexcluded is the one described in possibility "c" above, namely that the 45-kDa band results from irreversible partial unfolding of Pfu TIM in its tetrameric state, leading to a greater level of SDS binding. In other words, the mobility differences seen in Fig. 2A are reflective of the occurrence of subtle (irreversible) substructural unfolding events.

Evidence of slow partial unfolding and no refolding from circular dichroism

The gel electrophoretic experiments reported in Figs 1–3 are indicative of Pfu TIM being extremely stable to unfolding even in the presence of heat and chemical denaturing agents such as urea or GdnCl. To corroborate these data with CD spectroscopic measurements, we examined the CD spectra of Pfu TIM prior to heating (in the presence of 4 M urea) and contrasted it with the CD spectra of the protein at 99 °C in the presence of urea (following 5 min of incubation at this temperature). As Fig. 5A shows, 5 min of heating at 99 °C in the presence of this high concentration of urea results in a noticeable reduction in secondary structural content; however, it is also clear that there is no noteworthy reduction of structural content, and the protein population remains largely folded, supporting the view that unfolding occurs extremely slowly as suggested by the electrophoretic data. Also, as indicated by the gel filtration data, the protein remains multimeric. Essentially the same conclusion is indicated by plotting the changes in structural

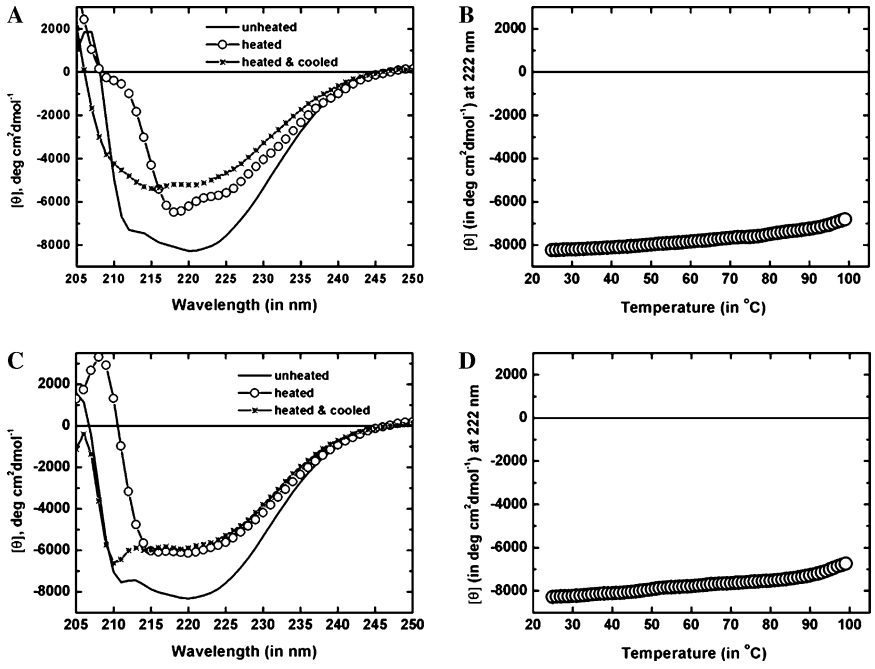

Fig. 5. No refolding of partially unfolded Pfu TIM. (A) CD spectra of Pfu TIM in the presence of 4 M urea prior to heating (25 °C), in the heated state (99 °C) and upon cooling back to room temperature (25 °C). (B) Nominal decrease in mean residue ellipticity at 222 nm, as a function of temperature (increased at a rate of 3 °C/min), in the presence of 4 M urea. (C) CD spectra of Pfu TIM in the presence of 2 M GdnCl prior to heating (25 °C), in the heated state (99 °C), and upon cooling back to room temperature (25 °C). (D) Nominal decrease in mean residue ellipticity at 222 nm, as a function of temperature (increased at a rate of 3 °C/min), in the presence of 2 M GdnCl.

content (Fig. 5B) with regard to the changes observed in mean residue ellipticity at 222 nm as a function of temperature. When the sample is cooled, there is no increase in structural content indicative of any refolding having occurred, as can be seen from the CD spectrum of the cooled protein shown in Fig. 5A. Essentially similar conclusions are indicated from the data for heating in the presence of 2 M GdnCl (Figs. 5C and D), except that in this case there is a nominal increase in ellipticity seen in the region of 210 nm upon cooling (indicative of partial recovery of helical content, which increases the negative signal at ~208 nm).

Broadly, it can be seen that the CD data are in agreement with the inferences drawn from electrophoretic and chromatographic analyses with regard to both (a) the slowness of the unfolding of Pfu TIM in the presence of heat and denaturants and (b) the irreversibility of such unfolding. Below, we show that mobility analysis and quantitation of conformers separated by gel electrophoresis can be used to derive plots of the kinetics of unfolding.

Transformation of gel band data into SDS binding or unfolding curves

Fig. 6 illustrates how the gel data can be transformed into three types of curves. The data in Fig. 6A (based on the gel shown in Fig. 2A) demonstrate how changes in relative mobility, obtained as a result of increased duration of incubation of Pfu TIM in low concentrations of denaturant at high temperature, reflect differences in SDS binding obtained through addition of SLB due to an apparent progressive increase in irreversible sub structural changes not amounting to a global unfolding of Pfu TIM. The curve illustrates how the protein population undergoes these subtle changes extremely slowly. It must be noted that the changes are not very easy to understand. For a two-state transformation, progressive changes in gel mobility (e.g., in urea gradient gels) or in elution volume during gel filtration are compatible with a situation in which two states interconvert rapidly in relation to the time scale of an unfolding experiment. When the time scale of the interconversion is large in relation to the time scale of the experiment, what is seen is not a progressive change but rather a two- state inversion of clearly distinct populations. In the particular case of the data in Fig. 2B, differences in the levels of SDS binding reflect differences in the occurrence of irreversible, subtle structural changes. When changes are irreversible, the question of interconversion of conformations does not arise and only a two-state inversion of populations is expected, rather than the progressive changes observed. This illustrates the usefulness of a gel electrophoretic approach in discovering novel phenomena that are too subtle to be picked up by other methods and that will require further investigation before they can be fully understood. With the data presented here, we have demonstrated, e.g., an increased susceptibility to SDS binding and unfolding by SDS that is not very apparent through the use of other methods.

With regard to the more gross sort of apparent two-state unfolding transitions, the data in Fig. 6B (based on the gel

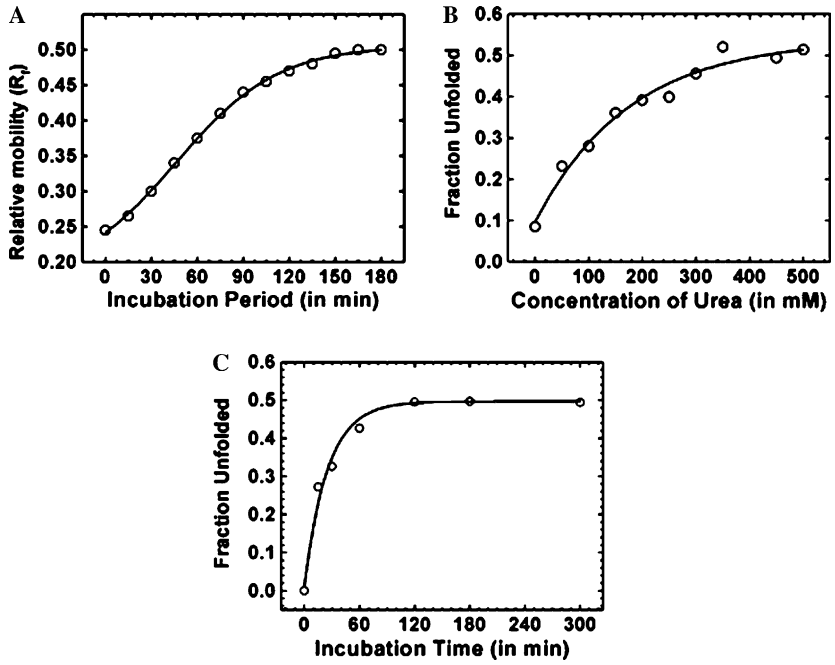

Fig. 6. Destabilization/unfolding profiles from gel data. (A) Kinetics of destabilization of Pfu TIM in the presence of 50 mM urea at 98 °C, plotted on the basis of changes in relative mobilities of bands as seen in lanes 2–14 of Fig. 2A. (B) Unfolding as a function of urea concentration, reckoned from densitometry of bands corresponding to lanes shown in Fig. 2B. (C) Kinetics of destabilization of Pfu TIM in the presence of 2 M GdnCl at 98 °C, reckoned from densitometry of bands corresponding to lanes plotted on the basis of changes in relative mobilities of bands as seen in lanes shown in Fig. 3B.

shown in Fig. 2B) demonstrate how densitometric quantitation of bands corresponding to folded and unfolded conformers can be used to generate conventional unfolding curves for varying denaturant (urea) concentrations of the type that one might create from other spectrometric studies. Of course, since the process does not reflect an equilibrium phenomenon, the value of this curve from the point of view of rigorous thermodynamic analyses remains suspect.

The data in Fig. 6C (based on the gel shown in Fig. 3B) demonstrate how densitometry of bands corresponding to folded and unfolded conformers can be used to generate a curve reflective of the kinetics of the unfolding process under a given set of conditions of temperature (98 °C) and GdnCl concentration (2 M). As is clear from the curve, these conditions fail to elicit complete unfolding of the population. This analysis of populations from direct visualization and quantitation offers advantages not available in spectroscopic methods.

Thus, it can be seen that this novel electrophoretic approach, which allows separation and quantitation of hyperthermophile protein conformers, has at least two distinct advantages. It helps in the detection of subtly different conformations, on the basis of differential SDS binding. It also helps in following the kinetics of irreversible unfolding through running of a gel after the unfolding experiment has been carried out, allowing actual separation of conformers. In cases where kinetically stable intermediates are formed, the method could also be expected to help in separating and quantitating such intermediates. We welcome the use of this method by other groups, using other hyperthermophile proteins. With regard to the study of Pfu TIM itself, we have established that the protein is extremely kinetically stable to unfolding and that it can be brought to an unfolding-competent state (vulnerable to unfolding by SDS) only very slowly, using a combination of physical and chemical means. We have now also seen this behavior with a few other hyperthermophile proteins that we are producing in *E. coli*.

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