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4.1 Introduction

Most proteins from hyperthermophiles are considered to unfold irreversibly (Klump H et al., 1992 and Higgins CL et al., 2002). We exploited this property to develop and demonstrate a novel gel electrophoresis-based method for the assessment of hyperthermophile protein stability. The recombinant Triose phosphate IsoMerase (TIM) from *Pyrococcus furiosus* (PfuTIM) described in the previous chapter was used as the subject of the study. The rationale behind the approach is described below.

The electrophoretic mobility of a protein on an SDS-PAGE can be directly correlated with its molecular mass. However, this relationship holds only if the protein happens to unfold fully and binds SDS to saturation prior to exposure to an electric field. If unfolding is incomplete for any reason, a protein might instead be expected to display an anomalous mobility (i) either because of poor binding of SDS (resulting from the non-exposure of hydrophobic residues due to incomplete unfolding) which reduces the net negative charge available on the protein during electrophoresis, or (ii) because compactness of conformation, associated with a smaller hydrodynamic volume, leads to lesser retardation of movement of the protein within the gel matrix. Exactly which of these influences ends up determining a protein’s mobility is, of course, likely to be dependent on the degree of survival of structure. If a ‘sufficient’ number of SDS molecules (corresponding to the protein’s size) were to bind to each protein molecule despite the incompleteness of unfolding, of course, a greater mobility than expected would be observed, because of the survival of a conformation of greater compactness than that of the fully unfolded protein. On the other hand, despite the compactness of conformation facilitating a smaller hydrodynamic volume, an ‘insufficient’ degree of binding of SDS could actually cause the protein to show lesser mobility than anticipated for the fully unfolded protein.

Preparation of protein samples for SDS-PAGE normally involves the use of a combination of two destabilizing influences to ensure the complete unfolding and binding of SDS: (i) chemical agents that denature and reduce the protein (i.e., SDS, and DTT/β-ME), and (ii) heating of the sample in the presence of the above agents, through boiling for a few minutes. We have found that whereas with mesophile-derived proteins, elimination of the heating step does not affect the mobility obtained (in most cases) because such proteins often, anyway, tend to be unfolded by the combination of
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SDS and a reducing agent, the situation can be very different with a hyperthermophile protein. A hyperthermophile protein can be expected to be much more stable (and therefore much more resistant to unfolding) than most mesophile proteins. In experiments in which the unfolding of PfuTIM has been attempted e.g., by use of a combination of heat and chemical unfolding agents such as guanidine hydrochloride (GdnCl) or urea, we show that elimination of the boiling step during preparation of samples for gel electrophoresis facilitates separation of populations of folded and irreversibly-unfolded molecules. Molecules that have failed to unfold through exposure to heat and GdnCl/urea remain tetrameric and poorly bound by SDS, and display extremely poor mobility on SDS-PAGE if they have not been boiled after addition of the SDS-PAGE Sample Loading Buffer (SLB). On the other hand, molecules that have been irreversibly unfolded, readily bind SDS and display the expected mobility, regardless of whether samples are boiled or not.

Thus, the elimination of the boiling step in sample preparation that causes folded PfuTIM to display a different mobility from unfolded PfuTIM, allows a facile assay of the unfolding kinetics of this hyperthermostable protein.

4.2 Results

4.2.1 Distinguishing between folded and unfolded PfuTIM

All experiments described below were performed with a working protein concentration of ~1.4 mg/ml in 50 mM potassium phosphate buffer, pH 7.4 with 300 mM KCl. All SDS-PAGE analyses were performed on 10 % or 12 % acrylamide gels. Figure 4.1a (lane 2) shows that PfuTIM 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 the SLB is added but samples are not subjected to boiling (lane 3), PfuTIM displays an anomalously low mobility. The same anomaly is shown by samples that have been boiled independently for 5 min, and cooled to room temperature, prior to the addition of SLB (lane 4), with no further boiling effected in the presence of SLB. Thus, a combination of heat (boiling for 5 minutes) 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 is seen as a faint band of anomalous mobility near
Figure 4.1 Fig. 4.1a shows the anomalous mobility of PfuTIM as described in the text. Fig. 4.1b compares whole cell extracts from control and transformed cells expressing PfuTIM. Figs. 4.1c and 4.1d represent control experiments with samples incubated in the presence of SDS alone and heat alone, respectively.
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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 even in the presence of SDS and boiling). An identical situation is obtained with the sample in lane 4 which was also not boiled after addition of SLB; that this sample was boiled for 5 minutes prior to the addition of SLB remains inconsequential to the results obtained because proteins from *P. furiosus* are, in any case, not expected to be unfolded by a temperature of 100°C, since this temperature is the optimal growth temperature of the organism and below the maximum survival temperature (Bryant FO and Adams MW, 1989). Figure 4.1b verifies the results shown in Figure 4.1a in the context of the whole-cell proteins of *Escherichia coli* (strain M15), the expression host used for the recombinant production of PfuTIM. The figure shows that PfuTIM (lanes 4 and 5) is the only detectable protein in the extract of the transformed *E. coli* strain which displays different mobilities depending on whether the samples were boiled or not, after addition of SLB. The control lanes display whole cell extracts that have been boiled (lane 2) and not boiled (lane 1). This is not surprising, since the proteins of *E.coli* are not hyperthermostable whereas PfuTIM is. Figure 4.1c shows the results of a control experiment in which samples were incubated in the presence of SLB for upto 10 days at 25°C (lanes 2-9 represent samples taken after 1, 3, 4, 5, 7, 8, 9 and 10 days of incubation respectively) to see whether the failure of SDS to unfold PfuTIM prior to electrophoresis was a kinetic effect. The survival of the anomalous mobility over this period establishes that it is not (at least over the experimental timescales). Similarly, Figure 4.1d shows the results of another control experiment in which samples were incubated at 98°C for upto 20 hours (lanes 2-7 depict samples taken after 0.08, 2, 5, 10, 15 and 20 h of incubation respectively), prior to cooling, addition of SLB and electrophoresis. These samples even were found not to have unfolded, suggesting that the result inferred from lanes 3 and 4 of Figure 4.1a, concerning the failure of heat or SDS alone to unfold PfuTIM, was not a simple kinetic effect.

### 4.2.2 Unfolding PfuTIM by a combination of heat and urea

Figure 4.2a shows that heating of PfuTIM at 98°C in the presence of 50 μM urea effects a gradual change in the protein’s mobility with time (lanes 2-14 represent samples taken after 0, 15, 30, 45, 60, 75, 90, 105, 120, 135, 150, 165 and 180 min of incubation respectively) owing presumably to the progressive irreversible ‘unfolding’
of small substructures allowing greater binding of SDS when SLB is subsequently added. Over a period of three hours of incubation, the mobility of PfuTIM 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 an ~25 kDa monomer. The same resultant migratory pattern (as in lanes 12-14 of Figure 4.2a) was found with 2 M urea, when samples were heated for only 1 hr (data not shown). This sample was then run on a Superdex-75 gel filtration column in the same buffer used for gel electrophoresis, mimicking the very conditions. It was seen (Figure 4.3) that the species migrating at an apparent molecular weight of 45 kDa runs exactly like the untreated protein, indicating that it may be a tetramer. Thus, the molecule moves faster by dint of a better SDS-binding ability. Figure 4.2b shows that incubation over the same three hours at 98°C in the presence of higher concentrations of urea (lane 1 represents 0 μM and lanes 3-11 represent 50, 100, 150, 200, 250, 300, 350, 450 and 500 μM urea respectively) progressively elicits an apparently two-state transformation of significant fractions of the protein population from the species displaying an apparent molecular weight of about 45 kDa to the completely unfolded monomer displaying a molecular weight of ~25 kDa. However, 500 μM urea fails to effect unfolding of the entire population. Figure 4.2c shows that such unfolding is achieved by raising urea concentrations further. Using an incubation time of 3 hours and different concentrations of urea (lanes 1-3 represent 0, 0.5 and 1 M and lanes 5-10 represent 1.5, 2, 2.5, 3, 3.5 and 4 M urea respectively), it is observed that a urea concentration greater than 2 M is required to unfold the entire population. A control experiment (Figure 4.2d) shows that incubation without concomitant heating for 12 hours in the presence of urea alone (i.e., at 25°C), using concentrations of urea ranging from 100 μM to 1 M (lanes 1-4 represent 0.1, 0.2, 0.3 and 0.4 M urea and lanes 6-11 represent 0.5, 0.6, 0.7, 0.8, 0.9 and 1 M urea respectively and lane 12 represents a heated control without denaturant), elicits no unfolding, demonstrating that the effects observed result from a combination of heat and urea. A control experiment (data not shown) was also performed where 1 M urea solution, and the protein were heated separately at 98°C, cooled, mixed and analyzed. Another such control was also performed, where the protein was heated alone at 98°C and mixed with 1 M urea and analyzed similarly, to rule out the formation of active radicals in heated urea solution or the unfolding of the protein by heat alone being the cause of the observed effect. The protein was found to run above 66 kDa in both of
Figure 4.2 Fig. 4.2a represents the effect of 50 μM urea and heat (98°C), for increasing periods over 0-3 h. Fig. 4.2b depicts the same experiment done for 3 h, with increasing concentrations of urea from 0-500 μM. Fig. 4.2c shows a further increase in urea concentration, i.e. 0-4 M. Fig. 4.2d shows a control, where samples were incubated at 98°C for 12 h in 100 μM-1 M urea.
Figure 4.3  Fig. 4.3 represents a gel filtration profile for a sample that has not been treated (control) and a sample that has been heated with 2 M urea for 1 h at 98°C.
these controls. The effect on protein structure was also studied using Circular Dichroism (CD) spectroscopy. Protein concentration used for all the CD experiments mentioned here, were kept at \( \sim 0.18 \) mg/ml. Figure 4.5a shows the CD spectra of PfuTIM with 4 M urea, i.e. the concentration that caused complete unfolding in 3 h as shown in lane 8 of Figure 4.2c. The spectra for the initial sample, heated sample and cooled back samples are as denoted on the graph. As can be seen, the protein loses structure, mostly \( \alpha \)-helix content as it is heated. The sample does not completely regain native structure even upon cooling. Figure 4.6a shows the decrease in ellipticity at 222 nm \( (\theta_{222 \text{ nm}}) \) during a controlled heating of the sample from 25°C to 99°C, at 3°C/min. The unfolding reaction is seen to be starting off only towards the later part of the spectrum.

4.2.3 Unfolding PfuTIM by a combination of heat and GdnCl

No unfolding was observed upon incubation with 4 M GdnCl alone at 25°C (data not shown). Even in the presence of 1 M GdnCl, PfuTIM remained essentially folded for up to 20 hours at 98°C as shown in Figure 4.4a (lane 1 represents 0 h control and lanes 3-9 represent 0.25, 0.5, 1, 2, 5, 10 an 20 h of incubation respectively). However, upon incubation at 98°C with a higher GdnCl concentration of 2 M, significant unfolding was achieved over shorter durations of incubation as shown in Figure 4.4b (lanes 1-3 represent 0, 0.25 and 0.5 h and lanes 5-8 represent 1, 2, 3 and 5 h of incubation respectively). Raising GdnCl concentration further to 4 M elicits unfolding of almost the entire population within 3 hours of incubation at 98°C as shown in Figure 4.4c (lane 1 represents 0 M control and lanes 3-10 represent 0.5, 1, 1.5, 2, 2.5, 3, 3.5 and 4 M GdnCl respectively). Unlike with urea, no partial structural changes were seen at low GdnCl concentrations, with the entire scheme of unfolding appearing to be two-state. Figure 4.5b shows the CD spectra of PfuTIM with 2 M GdnCl, i.e. the concentration that caused incomplete unfolding in 3 h as shown in lane 8 of Figure 4.4b. The spectra for the initial sample, heated sample and cooled back samples are as denoted on the graph. As can be seen, the protein loses structure, both \( \alpha \)-helix and \( \beta \)-sheet content as it is heated. The sample does not completely regain native structure even upon cooling. Figure 4.6b shows the decrease in ellipticity at 222 nm \( (\theta_{222 \text{ nm}}) \) during a controlled heating of the sample from 25°C to 99°C, at 3°C/min.
Figure 4.4  Fig. 4.4a represents the effect of 1 M GdnCl and heat with increasing lengths of time. Fig. 4.4b depicts the same experiment repeated with 2 M GdnCl with increasing durations of incubation upto 3 h. Fig. 4.4c is a further increase in GdnCl concentration upto 4 M with increasing incubation periods upto 4 h.
Figure 4.5  Fig. 4.5a represents CD spectra of PfuTIM in the presence of 4 M urea for unheated, heated and heated & cooled samples. Fig. 4.5b represents similar spectra collected in the presence of 2 M GdnCl.
Figure 4.6 Fig. 4.6a represents $\theta_{222\,\text{nm}}$ of PfuTIM in the presence of 4 M urea, heated from 25°C to 99°C. Fig. 4.6b represents similar $\theta_{222\,\text{nm}}$ collected in the presence of 2 M GdnCl.
The unfolding reaction is seen to be starting off towards the later part of the spectrum, as seen with 4 M urea.

4.2.4 Quantitation of the unfolding by densitometry

The unfolding of PfuTIM observed from the urea and GdnCl data was analyzed quantitatively using densitometry. The gel shown in Figure 4.2a was analyzed for the relative mobilities of the structurally destabilized species created by the effect of heat and a low urea concentration (50 μM). Figure 4.8 shows the result of such an analysis where the relative mobilities were plotted against time. The sigmoidal fit curve shows that the process is quite linear with a very low degree of cooperativity. Thus, PfuTIM is not cooperatively converted to a species that readily binds SDS and moves faster on the SDS-PAGE.

Figure 4.9a and b show the densitometric quantitation of the formation of the completely unfolded, highly SDS-bound form of PfuTIM from the native PfuTIM by the effect of urea and GdnCl, respectively, in combination with heat. The fraction unfolding is defined as the fraction unfolded/(fraction folded + fraction unfolded). This value is plotted against urea concentration in Figure 4.9a to obtain an equilibrium unfolding curve and against time (at a given GdnCl concentration) in Figure 4.9b to obtain kinetic data. Thus, it can be seen that the method is very useful in obtaining unfolding kinetics for local structural destabilization events that cause unfolding, where conventional methods fail to detect any destabilization.

4.2.5 PfuTIM is not unfolded either by cold or by a combination of cold and urea/GdnCl

Since PfuTIM 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 the electrophoretic assay to examine whether the protein would show any evidence of cold denaturation at low temperatures, as is observed for some hyperthermophile proteins (Sterner R and Liebl W, 2001). No unfolding whatsoever was observed over days of incubation at 6°C either in the absence, or presence of urea (4 M) or GdnCl (4 M), indicating that neither cold alone or a combination of cold and chaotropic agents effect unfolding of this protein as shown in Figure 4.7a (lanes 2-5 represent incubation in the absence of denaturants for 0, 1, 2 and 3 days respectively, lanes 6-9 represent
Figure 4.7  Fig. 4.7a shows protein samples treated with and without 4 M urea and 4 M GdnCl for up to 3 days. Fig. 4.7b represents samples treated with a combination of 3 M urea and 3 M GdnCl for up to 10 h.

Figure 4.8  Fig. 4.8 shows the effect of 50μM urea and 98°C on the relative mobility of PfuTIM with respect to time, based on the gel shown in Fig. 4.2a.
Figure 4.9 Fig. 4.9a shows the effect of 98°C on the unfolding of PfuTIM with respect to urea concentrations, where samples were incubated for a fixed time duration of 3 h (based on the gel shown in Fig. 4.2b). Fig. 4.9b shows the effect of 2 M GdnCl and 98°C on the unfolding of PfuTIM with respect to time (based on the gel shown in Fig. 4.4b)
incubation in the presence of urea for 0, 1, 2 and 3 days respectively and lanes 10-13 represent incubation in the presence of GdnCl for 0, 1, 2 and 3 days respectively). The experiments were also repeated with a mixture of denaturants i.e. 3 M urea and 3 M GdnCl for up to 10 h as shown in Figure 4.7b (lanes 2-6 represent incubation for 0, 1, 2, 5 and 10 h respectively). The experiments were also repeated at -20°C for up to 12 h, -70°C for up to 10 h and -196°C (in liquid nitrogen for 50 freeze-thaw cycles) without any detectable unfolding (data not shown).

4.3 Conclusions

The above set of experiments describes a novel method to study unfolding and to detect intermediates (more than 10 in number for PfuTIM as per Figure 4.2a) during the course of unfolding of a hyperthermophile protein. The robustness of these proteins was also established. The results also suggest that the combination of urea and heat causes a stepwise ‘teasing out’ of structure, causing an increased binding of SDS that results in an increased mobility on the SDS-PAGE. Subsequently, a complete denaturation of subunits (at higher urea concentrations or longer incubation times) facilitates an SDS-induced dissociation to monomers. The combination of GdnCl and heat, on the other hand, causes complete denaturation of the subunits under lower concentrations, which are then easily dissociated into monomers by SDS. Thus, it could be deduced that there are ionic interactions that play a major role in the stabilization of PfuTIM as urea (which is a non-ionic denaturant) is unable to effect denaturation at lower concentrations, as is found in chapter 3 also. None of the destabilizing conditions were able to effect unfolding alone.

The data obtained also shows that the method is able to detect subtle structural destabilizations that are not detected by spectroscopic methods. Thus, minor changes occurring by the combined action of physical and chemical denaturants, which are unable to cause global structural unfolding in a detectable fraction of the population is seen to manifest as a drastic change on an SDS-PAGE. The assay proves to be a novel and an improved method to observe and quantitate such minor and irreversible unfolding transitions.
4.4 References


