Is an intermediate state populated on the folding pathway of ubiquitin?

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Abstract In the last couple of years, there has been increasing debate as to the presence and role of intermediate states on the folding pathways of several small proteins, including the 76-residue protein ubiquitin. Here, we present detailed kinetic studies to establish whether an intermediate state is ever populated during the folding of this protein. We show that the differences observed in previous studies are attributable to the transient aggregation of the protein during folding. Using a highly soluble construct of ubiquitin, which does not aggregate during folding, we establish the conditions in which an intermediate state is sufficiently stable to be observed by kinetic measurements.

Keywords: Two-state; Three-state; Intermediate; 1-Anilinonaphthalene-8-sulfonate; Chevron plot; Rollover

1. Introduction

Recently, the presence and role of partially structured intermediate states on the folding pathways of small monomeric proteins has come under some scrutiny. In two cases, the folding of proteins thought to populate stable intermediates during folding has been questioned [1,2]. In the first case, the kinetics of unfolding and refolding of barnase have been reanalysed and good kinetic evidence provided that there is an on-pathway intermediate [3]. In the second case, the folding of the small 76-residue protein ubiquitin, which was originally reported to fold with three-state kinetics and to transiently populate an intermediate state under native-like conditions [4], was shown to fit well to a two-state model where only the denatured and native states are significantly populated [1].

One of the major difficulties in establishing whether an intermediate state is populated or not is the fact that (for small proteins) intermediate states are very transient species usually formed rapidly within the dead time of mixing of many experimental techniques such as stopped-flow spectroscopy. In these cases, it is often impossible to detect the intermediate directly. Ultrafast mixing and detection devices such as continuous flow, or perturbation methods such as temperature jump, which can monitor reactions on the microsecond to millisecond time scale, overcome some of the limitations of stopped-flow techniques and have been used to monitor the formation of intermediate states directly [5–9]. Although these are very powerful techniques, they require specialised instrumentation, the use of which is still limited. As a result of this, it is therefore common to employ indirect methods to detect and characterise intermediate states.

Pulsed–quenched H/D exchange techniques were one of the first methods used to obtain high-resolution information on the structure of folding intermediates [10–18]. In theory, this technique is extremely powerful as a means of establishing the presence of, and characterising, folding intermediates; in practice, however, the method has a number of limitations. In some cases, transient aggregation can occur during folding resulting in the protection of amide groups without populating an intermediate state [3]. In other cases, the pH of the labelling pulse may destabilise the intermediate state and result in reduced protection [19]. It has also been suggested that structure may form without the significant protection of the backbone amide groups [20].

The presence of intermediate states is often inferred from burst phases which are sometimes observed to occur within the dead-time of mixing in many folding experiments [8]. In these cases, there is a significant difference in the initial spectroscopic signal observed and that expected from the denatured state. However, this approach depends critically on determining the expected initial and final baselines corresponding to the denatured and native states with great accuracy. This can be difficult as the spectroscopic signal from the unfolded and native states can be very dependent on experimental conditions such as denaturant concentration, pH, temperature, etc. Usually a linear extrapolation method is used to estimate the baselines in native conditions from denaturing conditions. Recently, however, it has been shown that this method is not always valid [21,22].

A now commonly used approach to detect and characterise folding intermediates has been to measure the rate constants for unfolding and refolding as a function of denaturant concentration and to plot the logarithm of the rate constant versus denaturant concentration in what has become known as a chevron plot [23–25]. This approach is based on the seminal work of Tanford who established that there are linear free energy relationships between the free energies of the different folding states and denaturant concentration [26]. In general, for proteins which fold without populating an intermediate state both arms of the chevron plot are linear [24]. In comparison, deviation from linearity

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Abbreviations: ANS, 1-anilinonaphthalene-8-sulfonate; GdmCl, guanidinium chloride

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occurs at low concentrations of denaturant for proteins which begin to populate intermediate states under native-like conditions (rollover is observed) [25]. Several groups have used this approach to both detect and characterise intermediate states [4,27,28].

In recent years, however, it has become clear that there are limitations to this approach and that curvature or rollover in chevron plots may result from events other than the population of an intermediate state. In particular, it has been established that rollover can result from the transient aggregation of the protein during folding [29]. It has also been demonstrated that the rate constants can, in some cases, be sensitive to the exact method of analysis, dead time of the instrument and the presence of slow proline-isomerisation-limited refolding phases [1].

As well as considering the limitations in the different methods and approaches used to detect and characterise intermediate states, one also needs to bear in mind that these states may only be significantly populated, and therefore detectable, under certain experimental conditions. This is nicely illustrated by studies on the immunity family of proteins which have shown that, whilst some family members populate an intermediate state over a wide range of experimental conditions [27], an intermediate state is only observed for other members under a relative limited set of experimental conditions [30], demonstrating the importance of scanning a wide range of experimental conditions when characterising the folding kinetics of a protein.

In this study, we establish under what conditions an intermediate state is populated during the folding of the small protein ubiquitin. To do this, we have measured the kinetics of unfolding and folding of ubiquitin over a wide range of experimental conditions from pH 2.2 to 7.4 using both guanidinium chloride (GdmCl) and urea as chemical denaturants, and in the presence and absence of stabilising salts. In doing so, we have undertaken a comprehensive study of the factors that contribute to curvature in kinetic chevron plots.

2. Materials and methods

A non-tagged F45W mutant of ubiquitin (Met1-Gly76) was a kind gift from Andy Robertson. We also used a tagged F45W variant of ubiquitin, which has an additional seven-residue (GLVPRGS) N-terminal tail due to the cleavage of an affinity tag. This was expressed and purified as described elsewhere [31]. Buffers and denaturant solutions were prepared, and equilibrium experiments performed as described elsewhere [31]. The kinetics of unfolding and refolding were measured as a function of denaturant concentration using a combination of [denaturant]-jump, pH-jump and SDS/α-cyclodextrin-jump experiments all of which are described in detail elsewhere [31–33]. Data were analysed as previously described [31].

3. Results

3.1. Unfolding/folding kinetics of non-tagged F45W mutant of ubiquitin at pH 5.0

The kinetics of unfolding and folding of the non-tagged F45W fluorescent mutant of ubiquitin (Met1-Gly76) were measured at pH 5.0 under similar conditions to the original studies of Roder and co-workers [4,34] and the more recent studies of Sosnick and co-workers [1]. The results are shown in Fig. 1A (black closed circles).[1] In this case, significant curvature was observed at low concentrations of denaturant, which has previously been attributed to the population of an intermediate state [4]. In order to establish whether this curvature was due to the transient aggregation of the protein during folding, the dependence of the refolding rate constant on protein concentration was measured (Fig. 1B). It is clear from this study that significant transient aggregation occurs under these conditions even at very low protein concentrations [29]. These results explain the apparent contradiction in the folding kinetics previously reported for ubiquitin [1,4]. In these two studies, the experimental conditions were similar, however, the protein concentrations used were significantly different [1,4]. At high concentrations of protein the kinetics appear three-state [4], a consequence of the transient aggregation. At low protein concentrations, however, aggregation is significantly reduced and the kinetics become two-state [1].

3.2. Unfolding/folding kinetics of a soluble tagged-F45W mutant of ubiquitin at pH 5.0

We have also used an alternative construct of ubiquitin in which there are a few additional residues at the N-terminus due to the cleavage of an affinity tag (GLVPRGS-Met1-Gly76). The kinetics of unfolding and folding were measured at pH 5.0 (Fig. 1A, red circles) and compared with those obtained for the tagless F45W ubiquitin described above. In this case, we do not observe any rollover and the data fit well to a two-state model. In addition, we find that the protein is more soluble than the non-tagged F45W mutant used by the Roder and Sosnick groups and, by measuring the folding kinetics over a range of protein concentrations, we establish that there is no transient aggregation of this construct during folding (data not shown).

3.3. Unfolding/folding kinetics of non-tagged and tagged F45W ubiquitin at pH 2.2

Ubiquitin is acid stable and is fully folded even at pH 2.2. Kinetic measurements on both the non-tagged and tagged F45W variants were made at pH 2.2 and results are shown in Fig. 2A (using GdmCl as the denaturant). Fig. 2A shows clearly that there is a significant curvature at low concentrations of denaturant at pH 2.2 when GdmCl is used for both ubiquitin constructs. It has been known for some time that the stability of ubiquitin is dependent upon ionic strength, particularly at low pH when the protein carries a high net positive charge [35–37]. In order to establish whether the curvature in Fig. 2A is due to ionic strength effects, the refolding kinetics for our tagged F45W ubiquitin were also measured in urea (Fig. 2B). In this case, the plot becomes perfectly linear. Thus, it is clear that, at pH 2.2, unfavourable charge–charge interactions play a dominant role in stability and affect the folding

[1] To aid the reader the following colours and symbols are used throughout the figures: kinetic data obtained for the non-tagged F45W mutant of ubiquitin used in previous studies [1,4,34] are shown in black solid circles, kinetic data for the tagged F45W mutant of ubiquitin are shown in red. Solid circles indicate kinetic data measured in the absence of stabilising salts at 25 °C, solid squares indicate kinetic data measured in the absence of stabilising salts at 8 °C, open circles indicate kinetic data measured in the presence of 0.5 M KCl, and solid triangles indicate kinetic data measured in the presence of 0.4 M Na_2SO_4.

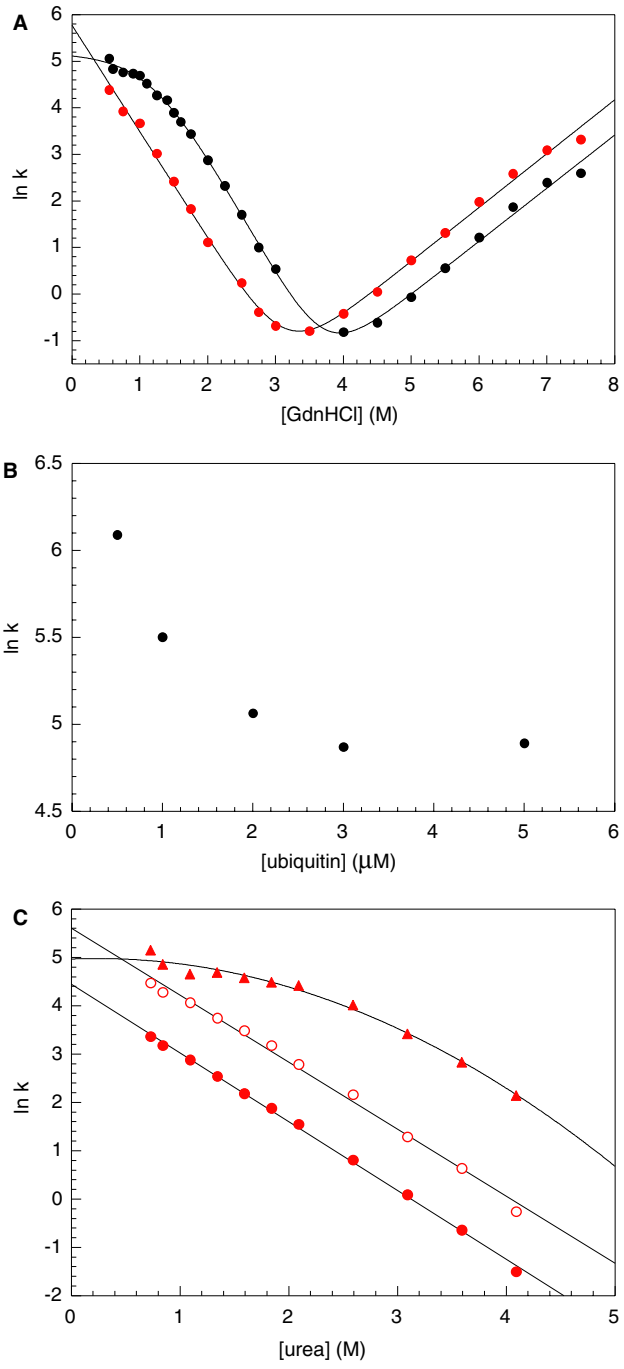

Fig. 1. Unfolding and refolding kinetics of ubiquitin in 25 mM acetate, pH 5.0. (A) Tagged-F45W (red open circles) and a non-tagged-F45W mutant (black closed circles). The latter is identical to the protein used in other studies [1,4]. Final protein concentrations were 2 μM. (B) Protein concentration dependence of the refolding rate constant for the non-tagged-F45W mutant at 0.5 M GdmCl at 25 °C. (C) Refolding kinetics of tagged-F45W in urea (red closed circles), in the presence of 0.5 M KCl (red open circles) and 0.4 M Na_2SO_4 (red closed triangles) at 10 °C. Final protein concentrations were 2 μM. In 0.4 M Na_2SO_4, the refolding rate constant in the absence of denaturant was measured using a SDS/α-cyclodextrin jump experiment [33]. Experimental methods were as described elsewhere [31–33]. The solid line shows the best fit of the data to a two-state [24] and three-state model [4].

kinetics when measured in GdmCl. We, therefore, measured the kinetics of unfolding and folding at pH 7.4 where the protein does not carry a high net charge.

3.4. Unfolding/folding kinetics of a soluble tagged-F45W mutant of ubiquitin at pH 7.4

The results obtained in GdmCl and urea are shown by the filled circles in Figs. 2C and D, respectively. No curvature is observed in GdmCl or urea in the absence of stabilising salts.

3.5. Unfolding/folding kinetics of a soluble tagged-F45W mutant of ubiquitin in the presence of stabilising salts

The above results suggest that, once transient aggregation and ionic strength effects are taken into account, the folding of ubiquitin is two-state. Several studies have shown that intermediate states can be populated on folding pathways by the addition of stabilising salts such as sodium sulfate [4,30]. Here, we investigated the effect of both potassium chloride and sodium sulfate on the folding kinetics of our tagged F4W construct over a range of conditions. The results obtained from measurements made in urea are the most straightforward to interpret as there are no ionic strength effects to complicate the analysis. The results obtained at pH 2.2 (Fig. 2B), pH 5.0 (Fig. 1C) and at pH 7.4 (Fig. 2D) are shown. At all three pHs, the logarithm of the folding rate constant versus denaturant concentration is linear in the absence of salts, and remains linear in the presence of 0.5 M KCl although there is an increase in the folding rate constants. In the presence of 0.4 M Na_2SO_4, however, significant curvature is observed. Importantly, in all these cases, a protein concentration dependence established that transient aggregation was not occurring under the conditions used (data not shown). Thus, it appears that an intermediate state is populated during the folding of ubiquitin when the protein is sufficiently stabilised by sodium sulfate.

3.6. Confirmation of a populated intermediate state by 1-anilinonaphthalene-8-sulfonate binding

In order to confirm that the curvature observed in the urea refolding plots of ubiquitin at pH 5.0 and 7.4 in the presence of 0.4 M Na_2SO_4 is due to the population of an intermediate state, additional refolding experiments were performed with the hydrophobic dye 1-anilinonaphthalene-8-sulfonate (ANS). ANS is known to bind to exposed clusters of hydrophobic residues frequently found in transient folding intermediates [38]. On binding to such a hydrophobic surface, ANS undergoes a large change in fluorescence and so is a sensitive probe of the formation of transient species during folding [38]. These experiments were undertaken with our soluble, tagged-F45W variant of ubiquitin, which we have shown is not susceptible to transient aggregation. Fig. 3A shows the change in ANS fluorescence during a refolding experiment at pH 5.0 where the final urea concentration is 0.72 M. Under these conditions, if the curvature observed in Figs. 1C and 2D is due to the population of an intermediate state, this intermediate should bind ANS and show a change in fluorescence. This is observed. At a final urea concentration of 3.6 M the amplitude is much reduced, see inset to Fig. 3A, as expected. The amplitude at 3.6 M urea is 1/80th of that measured at 0.72 M urea reflecting the fact that the intermediate state is destabilised relative to the denatured state with increasing concentrations of urea. Similar results are obtained at pH 7.4 (Fig. 3B). These results support the conclusion that curvature observed in the presence of the stabilising salt sodium sulfate results from the population of an intermediate state.

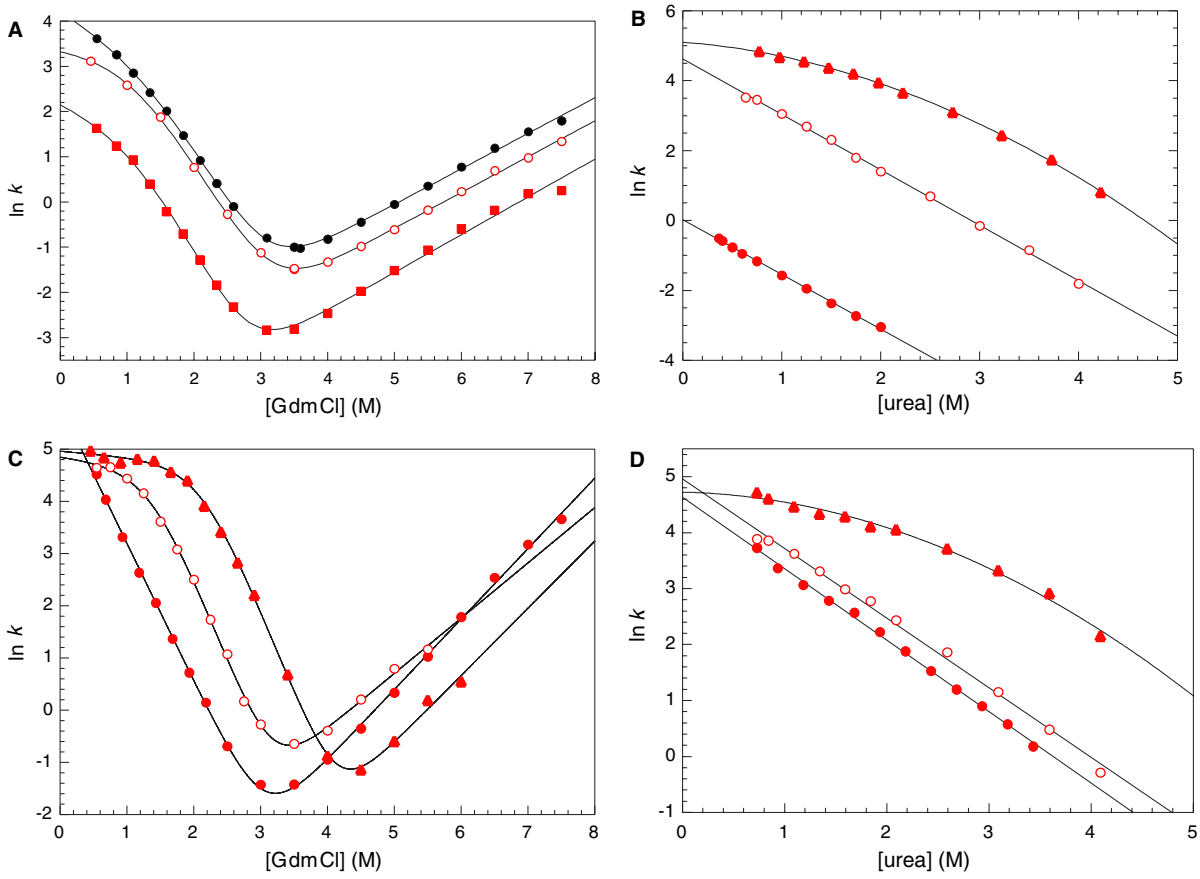

Fig. 2. Unfolding and refolding kinetics of non-tagged and tagged-F45W mutant of ubiquitin at 50 mM glycine, pH 2.2 (A,B), and 50 mM Tris–HCl, pH 7.4 (C,D). (A) Rate constants for unfolding and folding as a function of [GdmCl] for non-tagged-F45W at pH 2.2, 25 °C (black closed circles), tagged-F45W at 25 °C (red closed circles), tagged-F45W at 8 °C (red closed squares). The solid lines show the best fit of the data to a three-state model [4]. (B) Refolding kinetics of tagged-F45W in urea at pH 2.2 (red closed circles), in the presence of 0.5 M KCl (red open circles) and 0.4 M Na_2SO_4 (red closed triangles) at 10 °C. (C) Unfolding and refolding kinetics of the tagged-F45W mutant in GdmCl at pH 7.4, 25 °C in the absence (red closed circles) and presence of 0.5 M KCl (red open circles) and 0.4 M Na_2SO_4 (red closed triangles). The solid line shows the best fit of the data to a two-state [24] or three-state model [4]. (D) Refolding kinetics of tagged-F45W in urea (red closed circles), in the presence of 0.5 M KCl (red open circles) and 0.4 M Na_2SO_4 (red closed triangles) at pH 7.4, 10 °C. Final protein concentrations were typically 2 μM. Experimental methods were as described elsewhere [31,32]. In 0.4 M Na_2SO_4, the refolding rate constant in the absence of denaturant was measured using a SDS/α-cyclodextrin concentration jump [33].

3.7. Fast folding reactions: data analysis

Recently, it has been noted that curvature in chevron plots often occurs at low concentrations of denaturant where refolding rate constants are often high. In these cases, it has been proposed that rollover may result from errors in curve fitting as the rates approach the limit of stopped-flow instrumentation [1]. It has also been proposed that, at these limits, fast rates are masked by slower proline-isomerisation-dependent processes [1]. The refolding rate constants of our tagged-F45W–ubiquitin construct measured under our experimental conditions range between 0.15 and 250 s^{-1}. The fastest rates are approaching the limits of stopped-flow instrumentation when 1:10 ratios of mixing are employed. To establish the accuracy of the refolding rate constants, several additional experiments were performed. First, the dead-time of the instrument was measured under identical conditions to the refolding assays and was typically found to be 2 ms. Thus, even for the fastest folding events, more than 50% of the signal is observed and all refolding traces were analysed within the appropriate time range. Second, double-jump experiments were employed to ensure that refolding rate constants were not affected by proline isomerisation phases. The rate constants determined by double-jump experiments were within experimental error of the rate constants measured in single-jump mode (data not shown). We are confident, therefore, that curvature observed for the refolding of ubiquitin in the presence of sodium sulfate is not due to errors in data analysis.

4. Discussion

4.1. Causes of curvature in kinetic chevron plots

Transient aggregation. We have shown here that curvature in kinetic chevron plots of folding data can be attributed to many different factors. As has been previously reported [29], curvature may be due to the transient aggregation of the protein during folding even if the native state is soluble. These transient aggregation processes can occur even at very low protein concentrations; in the case of the U1A protein as low as 1 μM [29] and with ubiquitin at concentrations even lower than this (Figs. 1A and B). These concentrations are typical of those used in kinetic experiments and illustrate one limitation of this

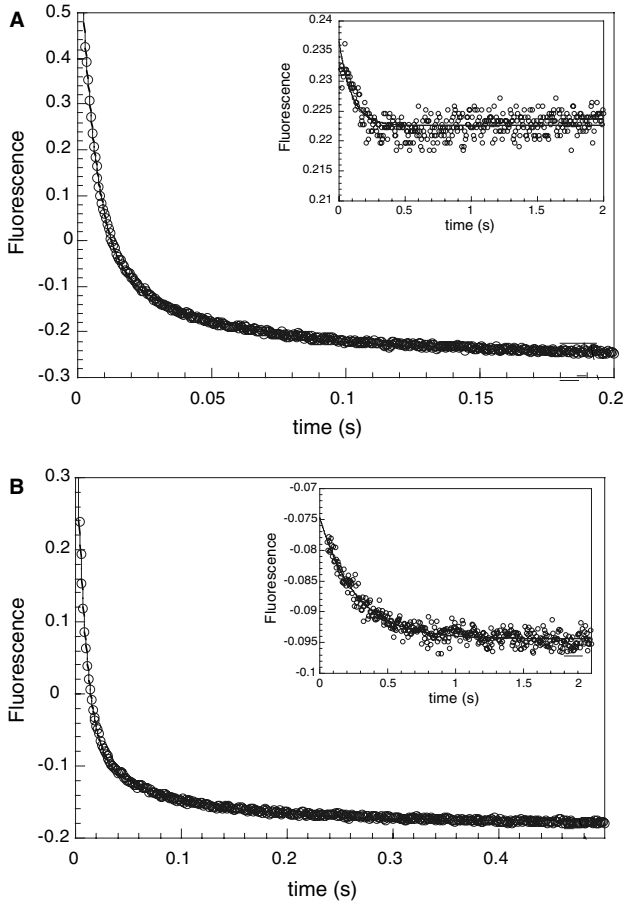

Fig. 3. Refolding kinetics of ubiquitin monitored by ANS fluorescence by [urea]-jump experiment. Final conditions are 2 μM tagged-F45W mutant of ubiquitin, 25 mM acetate, pH 5.0, 0.4 M Na_2SO_4 or 50 mM Tris–HCl, pH 7.4, 0.4 M Na_2SO_4 at 10 °C. (A) At pH 5.0 with a final urea concentration of 0.72 M (main plot) or 3.6 M urea (inset). The amplitudes and rate constants are 0.79 and 178 s^{-1} at 0.72 M and 0.01 and 11 s^{-1} at 3.6 M urea, respectively. (B) At pH 7.4 with a final urea concentration of 0.72 M (main plot) or 3.6 M urea (inset). The amplitudes and rate constants are 0.48 and 112 s^{-1} at 0.72 M and 0.02 and 3.8 s^{-1} at 3.6 M urea, respectively. Experimental methods are as described elsewhere [37].

type of analysis. The data presented here on different constructs of ubiquitin also highlight the sensitivity of aggregation processes to small changes in amino acid composition. Whilst the untagged F45W variant of ubiquitin is very sensitive to protein concentration and aggregates at very low concentrations, Fig. 1B, a construct which has a short additional N-terminal tag (GLVPRGS) shows no aggregation phenomenon.

Ionic strength effects. We have also established that curvature can result from changes in the ionic strength of the refolding buffer if GdmCl is used as the denaturant. In this case, the folding kinetics measured by [urea]-jump experiment can be quite different to those measured by [GdmCl]-jump (see Figs. 1 and 2). Electrostatic interactions are known to stabilise (or destabilise) the native states of proteins and such effects are sensitive to ionic strength [37]. If the native state of a protein is destabilised by electrostatic interactions, then high concentrations of salt (such as GdmCl) will stabilise the protein. At low concentrations of denaturant, the protein will therefore become less stable leading to a downward curvature in the chevron plot as observed for ubiquitin.

Hammond behaviour. It has also been suggested that curvature in both the unfolding and folding limb of chevron plots may result from changes in the position of the transition state as the relative energies of the transition and native state change, i.e., Hammond behaviour [39,40]. Can the curvature we observe for the refolding of ubiquitin in urea in the presence of sodium sulfate, Figs. 1 and 2, be attributed to such a phenomenon? Although we cannot exclude this possibility completely, it seems unlikely. We cannot measure the unfolding rates in urea due to the stability of the protein, however, no significant curvature is observed in the unfolding arm of the chevron plot in GdmCl. If the position of the transition state changes with the concentration of denaturant, we would expect to observe this as curvature in the unfolding limb of the chevron plots in GdmCl. This is not observed. Moreover, we have direct evidence from experiments with ANS that a partially structured intermediate state is populated under these conditions.

5. Conclusions

Our data are consistent with previously published work and explain why there is an apparent discrepancy in results from other groups [1,4]. These results, in conjunction with other studies [1,29,40], highlight the dangers inherent in interpreting curvature in chevron plots and the need for adequate control experiments, in addition to the need to probe a wide range of experimental conditions [30] in order to detect possible folding intermediates. Our results show that, in the absence of stabilising salts, no intermediate state is significantly populated during the refolding of ubiquitin consistent with recent results [1]. A partially structured intermediate state is populated on the folding pathway of ubiquitin only under conditions which stabilise the native state of the protein relative to the denatured state by some 2.5–3.0 kcal mol^{-1}. These results suggest that high-energy folding intermediates may exist on the folding pathways of other small proteins that show two-state kinetics. This is consistent with recent results on the kinetic intermediate identified on the folding pathway of acyl co-enzyme A binding protein which also shows two-state kinetics [41] and with recent work from the Kiefhaber group demonstrating that high-energy intermediates may be more common on folding pathways [42] than suggested by the number of proteins which fold with apparent simple two-state kinetics [43].

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