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Aggregation as the basis for complex behaviour of cutinase in different denaturants

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Abstract

We have previously described the complexity of the folding of the lipolytic enzyme cutinase from *F. solani pisi* in guanidinium chloride. Here we extend the refolding analysis by refolding from the pH-denatured state and analyze the folding behaviour in the presence of the weaker denaturant urea and the stronger denaturant guanidinium thiocyanate. In urea there is excellent consistency between equilibrium and kinetic data, and the intermediate accumulating at low denaturant concentrations is off-pathway. However, in GdmCl, refolding rates, and consequently the stability of the native state, vary significantly depending on whether refolding takes place from the pH- or GdmCl-denatured state, possibly due to transient formation of aggregates during folding from the GdmCl-denatured state. In GdmSCN, stability is reduced by several kcal/mol with significant aggregation in the unfolding transition region. The basis for the large variation in folding behaviour may be the denaturants' differential ability to support formation of exposed hydrophobic regions and consequent changes in aggregative properties during refolding.

Keywords: Cutinase; Folding scheme; Denaturant; Guanidinium thiocyanate; Aggregation

1. Introduction

Since Tanford's classic studies in the 1960s and 1970s [1–3], chemical denaturants such as urea and guanidinium chloride (GdmCl) have been very popular as tools to elucidate protein folding mechanisms. The key to their use lies in the empirically observed linear relationship between free equilibrium and activation energies of folding/unfolding versus denaturant concentration. Such linear relationships make it possible not only to extrapolate free energy values to zero molar denaturant, where unfolding cannot be measured directly, but also to propose kinetic folding models based on the variation of observed rate constants with denaturant concentration [4–7]. On a molar basis, GdmCl is approximately twice as potent as urea but also introduces somewhat more complex behaviour, due to its charge. This can apparently lead to stabilization of states which go unobserved in urea. Thus, GdmCl has been reported to induce the accumulation of partially folded states of protein disulfide isomerase [8] and carbonic anhydrase [9], which are not seen when unfolding is carried out in urea; however, when chloride ions in the form of NaCl are added to urea, an unfolding intermediate is in fact observed. An even more potent denaturant is guanidinium thiocyanate which has been used to denature e.g. exceptionally stable circularized variants of chymotrypsin inhibitor 2 [10] as well as compare effects of different denaturants [11,12].

Stable intermediates are typically inferred from equilibrium experiments when more than one spectroscopic transition is observed, or when transitions recorded by different techniques

Abbreviations: ANS, 8-anilino-1-naphthalenesulphonic acid; DLS, Dynamic light scattering

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do not coincide [13]. Although folding intermediates may be missed in equilibrium experiments because they do not accumulate as stable entities, they can be detected as transiently populated states through stopped flow techniques [14]. Typically the log of the measured rate constant k_{obs} is plotted as a function of denaturant concentration in a so-called chevron plot. Proteins folding according to a simple two-state system (D↔N) show a V-shaped chevron plot, in which the log of refolding rate constant declines linearly with denaturant concentration and vice versa for the unfolding rate constant. In contrast, a tell-tale signature of an intermediate is the "roll-over", in which the log of k_{obs} starts to deviate negatively from linearity at low denaturant concentrations, typically reaching a plateau level. This is an indication that the ground state from which folding occurs switches from the unfolded state to the intermediate state [4,6], leading to a slower refolding rate. However, such a roll-over can occur both through the formation of a productive ("on-pathway") as well as an unproductive ("off-pathway") intermediate (see Schemes 1 and 3 in Materials and methods) [4]. If the roll-over plateau flips over and assumes a positive slope, this is added evidence for an off-pathway intermediate which will have to unfold back to the denatured state to allow folding to occur [15]. These intermediates can also be regarded as physiological versions of the denatured state, i.e. the protein's response to the change in solvent conditions when it is rapidly diluted to lower concentrations of denaturant [6,16–18]. Due to their important but equivocal roles in folding, partially folded states have been the subject of intense investigation for many years.

The lipolytic enzyme cutinase is a 197-residue protein with a pI between 7.6 and 8.0 [19], which belongs to the superfamily of a/b hydrolase folds [20]. It shows complex folding and unfolding behaviour which together with its use in the food and chemical industry has attracted attention from several research groups. At pH 4.5, near-UV absorbance difference spectroscopy and fluorescence spectroscopy differ by ca. 0.1 M in the midpoints of their transitions in GdmCl, suggesting the presence of an intermediate state around the transition region of unfolding (1 M GdmCl) [21], which seems to have high affinity for the hydrophobic probe ANS. In addition, an unfolding intermediate also appears to accumulate transiently during unfolding at denaturant concentrations more than 1.5 M above the transition midpoint [22]. In the same study we observed a roll-over during refolding at low denaturant concentrations. The roll-over was very pronounced and seemed to be on the cusp of changing the sign of its slope, but as we were unable to follow unfolding all the way down to zero molar denaturant, we were not able to determine more unequivocally whether an off-pathway intermediate is formed. This subject is addressed in the present study, where we have extended the refolding concentration regime down to zero molar denaturant by measuring refolding from the pH-denatured state, obviating the need to dilute out denaturant to start refolding. We have also included the use of stabilizing salts to distinguish between the accumulation of on- and off-pathway intermediates. In addition, we compare the folding of cutinase in different denaturants (urea, GdmCl and guanidinium thiocyanate). This has allowed us to distinguish the different states that are populated during the folding process as well as revealing how sensitive the folding mechanism of a protein can be to the solvent.

2. Materials and methods

2.1. Materials

Ultrapure Guanidinium chloride (GdmCl) was from Life Technologies Inc. (Rockville, MA), guanidinium thiocyanate (GdmSCN) grade SigmaUltra (>99%) and all other chemicals were from Sigma-Aldrich (St. Louis, MO). All chemicals were of analytical or biological grade. An ANS stock solution was prepared by dissolving 30 mg ANS in 1 ml DMSO (dimethylsulfoxide) and adding 1 ml 25 mM acetate buffer pH 4.5 to give a final ANS stock solution of 50 mM. 197-residue cutinase from *Fusarium solani pisi*, cloned into the vector pFCEX1, was expressed in *E. coli* BL21 (DE3), purified to 99% purity, dialyzed against water and lyophilized [23]. The protein concentration was determined using ε_{280}=13,500 M^{-1} cm^{-1}.

2.2. Equilibrium denaturation experiments

All experiments were performed in 25 mM acetate buffer, pH 4.5 at 25 °C. This pH-value is far from the isoelectric point (pI=8.0) and thus reduces the potential for aggregation during folding or unfolding [24]. 8 M urea stock solutions were prepared fresh on a daily basis.

Equilibrium fluorescence studies at different denaturant concentrations were carried out using 5 μM cutinase with excitation of the single Trp at 298 nm and emission at 350 nm on an RTC2000 spectrometer (Photon Technology International, Lawrenceville, NJ) using slits of 5 nm for both excitation and emission. 298 nm was used to avoid contributions from tyrosine residues. When ANS was included, it was present at 40 μM; excitation was at 350 nm and emission was recorded at 475 nm. At each denaturant concentration, five scans were averaged to yield the final spectrum. No significant amount of photo-bleaching was observed due to the low slit values and the low absorbance at 298 nm. Equilibrium circular dichroism studies were performed on a Jasco J-715 spectropolarimeter (Jasco Spectroscopic Co., Hachioji City, Japan) with a Jasco PTC-348W1 temperature control unit. Six scans were averaged to yield the final spectrum. Ellipticity values at 223 nm were used, since ellipticity at this wavelength showed the greatest change upon denaturation. Absorption by urea prevented us from going below 215 nm.

The ellipticity and fluorescence values are analyzed using the linear extrapolation method [3,7], which is based on the relationship:

$$\log K_{D-N}^{urea} = \log K_{D-N}^{water} + m_{D-N}[urea] \quad (1)$$

where K_{D-N}=[D]/[N] is the equilibrium constant of unfolding (in water or at a specified urea concentration) and m_{D-N} is a parameter which reflects the degree of surface area buried in the native state, N, relative to the denatured state, D [3]. The larger the *m*-value, the greater the difference between N and D in exposed surface area. The advantage of this standard linear extrapolation approach is that it allows us to compare equilibrium and kinetic data, and thus test the consistency of kinetic interpretations (see below). Assuming a linear dependence of the pre- and post-transition baselines on urea concentration [7,25], we obtain the following equation for urea denaturation curves:

$$Y_{obs} = \frac{\alpha_N + \beta_N[urea] + (\alpha_D + \beta_D[urea]) * 10^{m_{D-N}([urea]-[urea^{50\%}])}}{1 + 10^{m_{D-N}([urea]-[urea^{50\%}])}} \quad (2)$$

where Y_{obs} is the observed signal, α_N and α_D denote the signal at 0 M urea for the native and denatured states respectively, β_N and β_D are the slopes of the baselines of the native and denatured states, respectively and [$urea^{50\%}$] is the urea concentration where 50% of the protein is denatured. As fluorescence signal we use the ratio of the emission at 350 and 310 nm, which increases by a factor of 6–8 upon unfolding. Non-linear least-squares regression analysis was carried out with the program Kaleidagraph, version 3.5 (Synergy