Kinetic folding mechanism of PDZ2 from PTP-BL

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PDZ domains represent a large family of protein-interaction modules associated with a variety of unrelated proteins with different functions. We report a complete characterization of the kinetic folding mechanism of a fluorescent variant of PDZ2 from PTP-BL, investigated under a variety of different experimental conditions. For this purpose, we engineered a fluorescent variant of this protein Y43W (called pseudo-wild-type, pWT43). The results suggest the presence of a high-energy intermediate in the folding of PDZ2, as revealed by a pronounced non-linear dependence of the unfolding rate constant on denaturant concentration. Such an intermediate may or may not be detectable depending on the experimental conditions, giving rise to apparent two-state folding under stabilizing conditions (e.g. in the presence of sodium sulfate). Interestingly, even under these conditions, three-state folding can be restored by selectively destabilizing the native-like rate-limiting barrier by one specific mutation (V44A). Finally, we show that data taken on pWT43 under different experimental conditions (e.g. different pH values from 2.1 to 8.0 or in the presence of a stabilizing salt) and also data on a site-directed conservative mutant can be rationalized in terms of a simple reaction scheme involving a single set of intermediates and transition states.

Keywords: chevron plot/Hammond effect/intermediate/protein folding/transition state

Introduction

PDZ domains (typically 80–100 residues) constitute a large family of protein-interaction modules that mediate protein–protein recognition by binding to short amino acid sequences (Fanning and Anderson, 1999). In fact, different classes of PDZ domains recognize specific C-terminal sequences (PDZ motifs) on a variety of protein substrates. The hundreds of unrelated proteins containing PDZ domains/motifs define highly complex interaction webs based on PDZ interactions (Dev, 2004; Kim and Sheng, 2004). Upon binding its target motif, PDZ domains undergo structural rearrangements that mediate signal transduction (Wilken et al., 2002). Furthermore, recent studies on murine PDZ2 from PTP-BL (Walma et al., 2002) in comparison with the alternative spliced form PDZas (Walma et al., 2004) have shown that the dynamics and stability of PDZ domains are crucial in controlling binding specificity, posing PDZ domains as useful experimental tools to investigate the relationships between folding and binding.

The structure of PDZ domains corresponds to a compact globular fold, composed of six β-strands and two α-helices; the six β-strands form two anti-parallel β-sheets stacked on to each other (Walma et al., 2002) (Figure 1). In this paper, we report a complete characterization of the kinetic folding pathway of a fluorescent variant of PDZ2 from PTP-BL, namely Y43W (pWT43), investigated under a variety of different experimental conditions. The results suggest the presence of a high-energy intermediate in the folding of PDZ2, which may or may not be detectable depending on the experimental conditions, giving rise to apparent two-state folding under stabilizing conditions. Recently, a hidden intermediate in the folding of PDZ3 from PSD-95 was identified by Bai and co-workers using hydrogen exchange (Feng et al., 2005). Examination of the structural differences between PDZ2 and PDZ3 suggests that the folding intermediate identified in this work is different from that characterized for PDZ3 and thus represents an additional high-energy species in the folding pathway of PDZ domains.

Materials and methods

Site-directed mutants were produced using a QuikChange site-directed mutagenesis kit (Stratagene). PDZ2 variants were purified as described previously (Walma et al., 2002). The buffers used were 50 mM sodium phosphate from pH 8.0 to 6.3, 50 mM sodium acetate from pH 5.5 to 3.8 and 50 mM sodium formate from pH 3.4 to 2.1. All reagents were of analytical grade.

Thermal unfolding

Equilibrium thermal denaturations were followed on a JASCO circular dichroism (CD) spectrophotometer using a

Fig. 1. Three-dimensional structure of PDZ2 from PTP-BL (pdb code: 1GM1). Residues Y43 (mutated in W for pWT43) and V44 (mutated to A in V44A) are indicated in stick representation.
0.1 cm quartz cuvette (Hellma). Protein concentration was typically 30 μM.

**Stopped-flow measurements**

Single mixing kinetic folding experiments were carried out on a Pi-star stopped-flow instrument (Applied Photophysics, Leatherhead, UK); the excitation wavelength was 280 nm and the fluorescence emission was measured using a 320 nm cut-off glass filter. In all experiments, performed at 25°C, refolding and unfolding were initiated by an 11-fold dilution of the denatured or the native protein with the appropriate buffer. Final protein concentrations were typically 1 μM. The observed kinetics were always independent of protein concentration (from 0.5 to 5 μM), as expected from monomolecular reactions without effects due to transient aggregation (Silow and Oliveberg, 1997). Unfolding experiments at low pH (≈3.8) were initiated by simultaneous pH jump and denaturant jump mixing (native protein in water).

Double jump interrupted unfolding experiments were performed on an Applied Photophysics SX18-MV stopped-flow instrument. Mixing ratios were 1:1 first mix and 1:1 second mix.

**Data analysis**

**Equilibrium experiments.** Assuming a standard two-state model, the urea-induced denaturation transitions were fitted to the equation

\[
\Delta G_d = \Delta G_0 - m_{D-N}(D - D_{50})
\]

where \(\Delta G_0\) is the free energy of folding in water and \(\Delta G_d\) at a concentration \(D\) of denaturant, \(m_{D-N}\) is the slope of the transition (proportional to the increase in solvent-accessible surface area on going from the native to the denatured state) and \(D_{50}\) is the midpoint of the denaturation transition. An equation which takes into account the pre- and post-transition baselines was used to fit the observed unfolding transition (Santoro and Bolen, 1988).

**Kinetic experiments.** Analysis was performed by non-linear least-squares fitting of single exponential phases using the fitting procedures provided in the Applied Photophysics software. The chevron plots were fitted by numerical analysis based on a three-state model as discussed in the Results section, using the Kaleidagraph software package. The logarithm of each microscopic rate constant was assumed to vary linearly with denaturant concentration (Jackson and Fersht, 1991).

**Results**

**Equilibrium unfolding of PDZ2**

In order to study the folding mechanism of PDZ2 from PTP-BL, we produced a fluorescent pseudo-wild-type, namely Y43W (pWT43) (Figure 1). Urea-induced equilibrium denaturation of pWT43 PDZ2 measured at 25°C, pH 7.0 in 50 mM sodium phosphate buffer by decrease in Trp emission is reported in Figure 2. The observed transition follows a simple two-state behavior, suggesting the absence of stable equilibrium intermediate(s) (Jackson and Fersht, 1991). The unfolding free energy in water derived from two-state analysis is 2.9 ± 0.2 kcal/mol displaying an m-value of 1.2 ± 0.1 kcal/mol/M. Thermal denaturation of pWT43 PDZ2, monitored by far-UV CD at several pH values, is also reported in Figure 2. The change in heat capacity, which is related to the amount of hydrophobic area that becomes solvent exposed on unfolding (Myers et al., 1995), was estimated indirectly from the variation of \(\Delta H_{D-N}\) and \(T_m\) at different pHs (data not shown). Owing to the low stability of pWT43, the unfolding \(\Delta C_p\) could only be determined over a narrow temperature window (~10°C) and should therefore be interpreted with caution. However, the estimated \(\Delta C_p = 1200 ± 150\) cal/mol.K is consistent with the value expected for a protein of this size (Myers et al., 1995) and successfully predicts the onset of cold denaturation, which is observed at low pH where the protein is partially unfolded at low temperatures (see pH 3.8 and 3.4 in Figure 2). Based on \(\Delta C_p\) and \(T_m\), it was possible to calculate the stability parameters for thermal denaturation. Importantly, the folding stabilities of pWT43 at different pH values
calculated at 25°C from thermal unfolding were in very good agreement with the values extrapolated from the analysis of urea-induced denaturation, confirming the two-state nature of the equilibrium unfolding of this small domain.

**Folding and unfolding kinetics**

The folding and unfolding kinetics of pWT43 PDZ2 were investigated at several pH values ranging from 2.1 to 8.0 and at 25°C. In all cases, folding and unfolding time courses were both fitted satisfactorily to a single exponential decay at any final denaturant concentration (Figure 3, top). Furthermore, although the native structure of PDZ2 displays two proline residues in a \( \text{trans} \) conformation, double mixing interrupted unfolding experiments (Kiefhaber et al., 1990) did not reveal the presence of slow proline \( \text{cis} \text{–trans} \) isomerization events (up to 50 s delay time; Figure 3, bottom).

A semi-logarithmic plot of the observed refolding rate constant versus denaturant concentration (chevron plot) measured at various pH values is reported in Figure 4. Interestingly, whereas the logarithm of the observed refolding rate constant decreases linearly with increasing denaturant concentration (as seen at pH > 5.5), the observed unfolding rate constants present a downward curvature as a function of urea (roll-over effect). Although this effect has been classically

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**Fig. 3.** Folding kinetics of pWT43 PDZ2. Top: refolding and unfolding time courses of pWT43 PDZ2 measured at 0.5 and 7.8 M urea, respectively. Lines are the best fit to a single exponential decay. Bottom: interrupted unfolding experiment measured at pH 7.0 in 50 mM sodium acetate and 25°C. Native molecules were unfolded in a first 1:1 mixing step with high denaturant concentration (final [urea] = 3.5 M); unfolded molecules were then refolded in a second 1:1 mixing step at various delay times, diluting urea with buffer in the presence of stabilizing salt (final [urea] = 1.75 M, 0.4 M sulfate). The observed amplitudes, reflecting the fraction of molecules in the \( \text{trans} \)-proline denatured conformation, are plotted as a function of delay times. Satisfactory fit to a single exponential decay \( k_{\text{obs}} \approx 0.58 \pm 0.03 \) indicates lack of a significant amount of \( \text{cis} \)-proline denatured conformation.

**Fig. 4.** Chevron plot and amplitude analysis of pWT43 PDZ2. Top: semi-logarithmic plot of the observed folding/unfolding rate constant versus denaturant concentration (chevron plot) measured at various pH values. The lines are the best fit to a three-state model as formalized in Equation 2; calculated parameters are listed in Table I. Bottom: urea dependence of the observed unfolding amplitude of pWT43 PDZ2 measured at pH 7.0 and 25°C. The line is the best fit to a two-state model (m-value, 1.1 ± 0.1 kcal/mol M; \([\text{urea}]_0, 2.30 \pm 0.05 \) M). Inset: observed initial (open circles) and final (filled circles) fluorescence signals of the monophasic unfolding reaction measured at pH 7.0 and 25°C as a function of denaturant concentration. The initial fluorescence (native state) displays a simple linear dependence on [urea], suggesting the absence of detectable burst-phase unfolding events (see text).
Experimental data were fitted to a three-state model as formalized in Equation 2. Standard errors are reported.

In theory, the kinetics of a two-step reaction should be fitted to the two roots of a quadratic equation, as shown previously for Int7 (Capaldi et al., 2001), HT and TT cyt c552 (Travaglini-Allocatelli et al., 2003, 2005) and the FF domain (Jemth et al., 2004). In many cases, including pWT43 PDZ2, only one relaxation rate can be observed experimentally, which jeopardizes a quantitative curve fitting. Two approximations have been introduced to describe the folding pathway of such multi-state systems. On the one hand, the intermediate is assumed to be in a fast pre-equilibrium with one of the ground states (the native state in the case of the unfolding reaction). Under such conditions, the curvature reflects a decrease in intermediate stability with respect to the native state on decreasing the denaturant concentration (Parker et al., 1995). This yields the following equation:

\[ k_{\text{obs}} = k_F + \frac{k_U}{1 + k_{\text{IN}}/k_{\text{UN}}} \]  
(1)

Alternatively, the intermediate is assumed to be at steady state and at low (approximately zero) concentration (Khan et al., 2003; Sanchez and Kiefhaber, 2003a). In this case, the roll-over is essentially caused by a change in rate-limiting step:

\[ k_{\text{obs}} = k_F + \frac{k_U}{\left(1 + k_{\text{IN}}/k_{\text{UN}}\right)} \]  
(2)

It should be noted that both approximations lead to very similar solutions with a partition factor describing alternatively either \( K_{\text{IN}}^b \) or \( k_{\text{IN}}/k_{\text{UN}} \) (i.e. reflecting the difference between the activation barriers for the intermediate to revert to the reagents rather than proceeding to the products) being kinetically indistinguishable on the basis of the analysis of rate constants only. Accumulation of an unfolding intermediate prior to the main rate-limiting barrier (as postulated in Equation 1) implies multiphasic unfolding kinetics, as described previously for a variety of different proteins (Kiefhaber, 1995; Khorsanizadeh et al., 1996; Eaton et al., 1997; Shastry and Roder, 1998; Bai, 1999; Ferguson et al., 1999; Capaldi et al., 2001; Teilm et al., 2002; Khan et al., 2003; Jemth et al., 2004). In the case of PDZ2, the unfolding free energy and \( m \)-value derived from analysis of the urea dependence of the amplitude of the mono-phasic unfolding reaction are in very good agreement with the parameters calculated from the chevron plot and the equilibrium titration measured under the same experimental conditions (Figure 4). Furthermore, the initial fluorescence of the observed unfolding time courses displays a simple linear dependence on [urea] (Figure 4, bottom, inset), indicating the absence of detectable burst phase unfolding events. On the basis of these observations, we suggest the absence of a low-energy populated intermediate in the unfolding pathway of PDZ2 and conclude that the unfolding roll-over effect is due to a change in rate-limiting step (as postulated in Equation 2).

The observed chevron plots measured at different pH values (Figure 4) were fitted both independently and globally to Equation 2 with shared kinetic \( m \)-values (Table I and Figure 4, top). Parameters calculated from global analysis allow the identification of the relative position of the two activation barriers along the reaction coordinate (Tanford \( \beta \)-value), resulting in a \( \beta \)-value of 0.53 ± 0.03 for the unfolded like transition state TS1 and 0.89 ± 0.03 for the native-like activation barrier TS2. Independent fitting of each individual chevron plot leads to very similar values. Comparison between global and free fitting of the data set, in order to probe possible shifts of the reaction barriers with changing stability of the ground states (Hammond effect) (Hammond, 1955; Matthews and Fersht, 1995; Sanchez and Kiefhaber, 2003b), is discussed below.

**Effect of a stabilizing salt**

Certain inorganic salts, such as phosphates and sulfates, favor compact protein conformations because of preferential exclusion from the protein surface; this makes them potent stabilizers of both the native and partially folded states (Timasheff, 1993). The folding and unfolding kinetics of pWT43 PDZ2 were studied at pH 7.0 in the presence of 0.4 M sodium sulfate; under such conditions, pWT43 PDZ2 is stabilized by ~2.5 kcal/mol. (Un)folding kinetics reported in Figure 5 show that, in the presence of the stabilizing salt, the chevron

<table>
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<tr>
<th>pH</th>
<th>( k_F ) (s(^{-1}))</th>
<th>( m^h ) (kcal/mol-M)</th>
<th>( k_{\text{IN}} ) (s(^{-1}))</th>
<th>( m^h ) (kcal/mol-M)</th>
<th>( K_{\text{part}} )</th>
<th>( m_{\text{part}} ) (kcal/mol-M)</th>
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<tr>
<td>8.0</td>
<td>3.08 ± 0.01</td>
<td>0.71</td>
<td>0.018 ± 0.005</td>
<td>0.63</td>
<td>0.020 ± 0.005</td>
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<tr>
<td>7.0</td>
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<td>0.71</td>
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<td>0.018 ± 0.005</td>
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<tr>
<td>6.5</td>
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<td>0.028 ± 0.004</td>
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<td>0.046 ± 0.01</td>
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<td>4.7</td>
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<td>0.38 ± 0.04</td>
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<td>0.075 ± 0.01</td>
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<tr>
<td>3.8</td>
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<td>0.71</td>
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<td>0.15 ± 0.01</td>
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</tr>
<tr>
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<td>5.75 ± 0.04</td>
<td>0.63</td>
<td>0.118 ± 0.002</td>
<td>-0.48</td>
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<tr>
<td>3.0</td>
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<td>12.7 ± 0.1</td>
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<td>0.19 ± 0.01</td>
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<tr>
<td>2.8</td>
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<td>0.71</td>
<td>17.9 ± 0.2</td>
<td>0.63</td>
<td>0.23 ± 0.01</td>
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<tr>
<td>2.1</td>
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<td>0.71</td>
<td>44.8 ± 0.1</td>
<td>0.63</td>
<td>0.26 ± 0.008</td>
<td>-0.48</td>
</tr>
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</table>

Experimental data were fitted to a three-state model as formalized in Equation 2. Standard errors are reported.

*The protein was too unstable to determine folding parameters.

*Values obtained from global fitting analysis of the whole experimental data set carried out at different pH values. Errors were estimated to be ±0.05 kcal/mol-M by calculating the standard deviation of the \( m \)-values obtained by fitting each data set individually.

*The partitioning factor \( K_{\text{part}} \) and its relative \( m \)-value refer to the ratio \( k_{\text{IN}}/k_{\text{UN}} \) as formalized in Equation 2.
Discussion

Kinetic folding pathway of PDZ2 from PTP-BL

Protein folding is a complex process involving the formation of a very large number of weak non-covalent bonds. Nevertheless, an increasing number of studies have shown that the denatured chain apparently folds to its native conformation in a surprisingly simple way, involving a limited number of intermediates (Daggett and Fersht, 2003). Identification of the minimal kinetic mechanism describing the folding of a protein is a crucial task in addressing the molecular details of the folding reaction.

In this paper, we propose a kinetic folding mechanism of PDZ2 from PTP-BL applicable under a variety of experimental conditions. Careful investigation of folding and unfolding kinetics of a fluorescent pseudo-wild-type, pWT43, at different pH values reveals the presence of an unstable unfolding intermediate, as detected from the roll-over effect in the unfolding arm of the chevron plot (Figure 4). Furthermore, quantitative analysis of the observed amplitudes for the mono-phasic reaction as a function of urea suggests that such an intermediate is a high-energy species, never accumulating under the experimental conditions explored. It should be pointed out that our experimental results might be compatible with an alternative model involving a low-energy populated intermediate whose fluorescence properties should, however, be identical with those observed for the native state.

The complex dependence of the unfolding rate constants on denaturant concentration allows us to estimate the position in the folding reaction coordinate of two different transition states (β-value of 0.53 ± 0.03 for TS1 and 0.89 ± 0.03 for TS2). Interestingly, in the case of PDZ2, folding can be easily tuned from a three-state to an apparent two-state model displaying a classical V-shaped chevron plot (Figure 5). In particular, by stabilizing the late transition state TS2 with sodium sulfate, this activation barrier is never rate limiting and becomes kinetically silent. Furthermore, folding kinetic experiments on the V44A mutant show that three-state kinetics can be then restored by destabilizing selectively the native-like transition state TS2 with respect to the unfolded-like transition state TS1.

Finally, we show that data taken either under different experimental conditions (e.g. different pH values or in the presence of a stabilizing salt) or with site-directed conservative mutants can be rationalized in terms of a simple reaction scheme involving a single set of intermediates and transition states. In this context, apparently different folding kinetics can be reconciled in terms of (de)stabilization of the relative activation barriers and ground states.

Chevron plots: global analysis versus Hammond effect

The position of the transition state along the reaction coordinate depends on the relative stabilities of reactants and products. Therefore, destabilization of the reagent shifts the transition state to become closer in structure to the destabilized state (Hammond effect) (Hammond, 1955). This effect can be monitored by measuring changes in observed β- or m-values upon destabilization of the native state, for example by changes in pH or by site-directed mutagenesis (Matthews and Fersht, 1995; Sanchez and Kiefhaber, 2003b; Fersht, 2004; Hedberg and Oliveberg, 2004). In the case of protein folding, it has been observed (Scott et al., 2004) that the complexity of the rate constant dependences on denaturant concentrations may require global analysis of different data sets (corresponding to different native stabilities) to extract reliable kinetic parameters. Global analysis generally assumes that changing the experimental conditions alters the stability of the ground states without altering the relative position of the folding transition state(s) and their β-value(s), denying a priori the presence of any Hammond effect, and may therefore be incorrect in theory.

In the case of PDZ2, we analyzed the experimental results using both the global analysis and a free fitting procedure.
stabilities (Gianni et al. 1999). Denaturant concentration: and unfolding reaction display a quadratic dependence on the logarithm of the microscopic rate constants for the folded state, thus becoming rate limiting. Under such conditions, a greater extent than the structures more similar to the denaturant, the structures closer to the native state are destabilized to a greater extent than the native state can be represented as a broad ensemble of structures (Ternstrom et al., 1999). Following this view, the folding transition state can be represented as a broad ensemble of structures along the reaction coordinates (Figure 7). On addition of denaturant, the structures closer to the native state are destabilized to a greater extent than the structures more similar to the denatured state, thus becoming rate limiting. Under such conditions, the logarithm of the microscopic rate constants for the folding and unfolding reaction display a quadratic dependence on denaturant concentration:

\[ \ln k_F = \ln k_W^F + m_F[\text{urea}] + m_F^2[\text{urea}]^2 \] (3)

\[ \ln k_U = \ln k_W^U + m_U[\text{urea}] + m_U^2[\text{urea}]^2 \] (4)

Furthermore, by applying the two-state and linear free energy dependence assumptions, one can postulate a symmetrical curvature of the folding and unfolding arm (Ternstrom et al., 1999):

\[ \frac{\partial \Delta G_{i-j}}{\partial [\text{urea}]^2} = 0 \] (5)

\[ m_F = m_U \] (6)

In the case of PDZ2 pWT43, the broad energy barrier model satisfactorily describes each individual chevron plot observed under different experimental conditions (data not shown). Interestingly, the calculated \( \beta_T \) at pH 7.0 in the presence of 0 and 8 M urea (0.53 ± 0.05 and 0.85 ± 0.08) are very similar to the values obtained from the sequential barrier model. These results are not surprising as it has recently been observed by Scott and Clarke (Scott and Clarke, 2005) that the broad energy barrier and sequential barrier model may lead to very similar conclusions even if challenged with a large number of site-directed mutants. However, it should be pointed out that, in the case of PDZ2 pWT43, the chevron plot can be turned into a simple V-shape, e.g. limited by a single, narrow, energy barrier, by addition of stabilizing salt. While this observation by itself may be easily described with the sequential barrier model (see above), it would imply substantial distortion of the folding free-energy profile in the case of the broad energy barrier model, thus posing it as less plausible for the folding of pWT43.

**Comparison with the folding of PDZ3 from PSD-95**

A powerful approach to elucidate the relationships between sequence information and folding mechanism is to study the folding kinetics of different proteins that share the same fold but differ in primary sequence. Several studies indicate that the overall folding mechanism is conserved within a fold family and common features can be identified even when apparently different folding mechanisms are observed (Grantcharova et al., 1998; Martinez et al., 1998; Ferguson et al., 1999; Riddle et al., 1999; Friel et al., 2003; Gianni et al., 2003; Travaglini-Alcocatelli et al., 2004).

The folding pathway of PDZ3 from PSD-95 has recently been studied by native hydrogen exchange and fluorescence, using the fluorescent variant F41W (Feng et al., 2005) homologous to the variant pWT43 of PDZ2 described in this paper. PDZ3 presents the typical PDZ fold but includes an additional \( \alpha \)-helix and an additional \( \beta \)-sheet at the N- and C-termini of the protein deriving from the expression vector (Doyle et al., 1996; pdb code: 1BE9). Quantitative analysis of hydrogen exchange kinetics reveals the presence of a hidden folding intermediate...
in PDZ3 folding. Furthermore, by comparing the different unfolding m-values obtained by NMR spectroscopy and fluorescence, Bai and co-workers concluded that such a folding intermediate is a low-energy species that should be populated at equilibrium (Feng et al., 2005). Interestingly, by analyzing the hydrogen exchange kinetics of individual residues of PDZ3, the authors proposed that the structure of the folding intermediate resembles that typical of the native PDZ fold, except for the N- and C-terminal regions, which are unfolded. This result was further confirmed by producing a deletion mutant of the C-terminal portion which unfolds the additional α-helix and an additional β-sheet at the N- and C-termini of the protein.

The folding pathway of PDZ2 from PTP-BL described here differs from the folding of PDZ3 proposed by Bai and co-workers. In the case of pWT43 PDZ2, the unfolding intermediate never accumulates and the complex unfolding kinetics are interpreted based on a change in rate-limiting step upon protein destabilization. It is important to note that PDZ2 lacks the additional α-helix and β-sheet at the N- and C-termini present in PDZ3, thus presenting per se the structure proposed by Bai and co-workers for the PDZ3 intermediate (Feng et al., 2005). We suggest, therefore, that the high-energy intermediate identified in this work represents an additional species in the folding of PDZ domains. Such an intermediate might be kinetically silent in the overall folding pathway of PDZ3 under the conditions investigated by Feng et al. More focused comparative studies on different PDZ variants are required to assess whether the high-energy intermediate identified in this work is common to the whole PDZ family, as observed in the case of the c-type cytochromes (Travaglini-Allocatelli et al., 2004).

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References

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