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A conserved folding mechanism for PDZ domains

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Abstract **An important question in protein folding is whether the folding mechanism is sequence dependent and conserved for homologous proteins. In this work we compared the kinetic folding mechanism of five *p*ostsynaptic density protein-95, *d*isc-large tumor suppressor protein, *z*onula occludens-1 (PDZ) domains, sharing similar topology but having different primary structures. Investigation of the different proteins under various experimental conditions revealed that the folding kinetics of each member of the PDZ family can be described by a model with two transition states separated by an intermediate. Moreover, the positions of the two transition states along the reaction coordinate (as given by their β_T-values) are fairly constant for the five PDZ domains.**

Keywords: Protein folding; Folding mechanism; PDZ domain; Kinetics

1. Introduction

At first sight the protein folding reaction appears enormously complex with hundreds of non-covalent interactions forming simultaneously. But, protein folding can be dissected like any chemical reaction: identify and characterize substrates, products and intermediates, and determine their pathway of interconversion and the associated rate constants. A powerful approach to elucidate the relationships between sequence information and folding mechanism is to study proteins which differ in sequence but share the same overall fold. The strategy assumes that general correlations between aminoacid sequences and folding pathways may be extrapolated by comparing folding processes for different members of a given protein family. Such studies have shown that the overall folding mechanism is generally conserved within a fold family, and hidden common features may be unveiled even when apparently different folding mechanisms are observed. For example, in the case of the homeodomain superfamily [1], where the folding processes may span from pure nucleation–condensation [2] to framework [3] mechanisms, the structure of the transition state is rather conserved [4]. Similarly for the bacterial immunity proteins Im9 and Im7, which appear to fold by two-state or three-state mechanisms [5], the late transition state ensembles of the two proteins have similar properties [6].

It has been shown previously that several proteins, including small single domain proteins, may accumulate obligatory folding intermediates [7,8]. Under such circumstances it is interesting to compare the structural properties of intermediate states, as well as the intervening transition states, when the amino acid sequence is varied but the three-dimensional structure is relatively unchanged [9]. In this study we have analysed and compared the kinetic folding mechanisms of four related but distinct *p*ostsynaptic density protein-95, *d*isc-large tumor suppressor protein, *z*onula occludens-1 (PDZ) domains together with one previously published [10,11]. All the proteins considered display the canonical PDZ fold [12]. Pairwise comparisons between the sequences of the PDZ domains give 25–50% identity but only 12 residues are conserved among all five domains (Fig. 1). We found that, despite this low identity and an apparent folding complexity, the folding reactions for PDZ domains can be explained by a model with an intermediate and two transition states that are rather conserved with regard to their positions along the folding reaction coordinate.

2. Materials and methods

2.1. PDZ constructs

The following PDZ domains were expressed as His-tagged proteins (MHHHHHPRGS-etc): PSD-95 PDZ1 (61–151), PSD-95 PDZ2 (155–249), and PSD-95 PDZ3 (309–401) (numbers in parenthesis refer to residue numbers in the parental human full-length PSD-95α without exon 4b, i.e. the same numbering as used in Doyle et al. [12] and Tochio et al. [13]). The PSD-95 PDZ domains will be referred to as PDZ1, PDZ2 and PDZ3, respectively. The pdb codes for the solved structures of these three PDZ domains are: PDZ1, 1IUO [14] (the numbering in the pdb file is 1–91), PDZ2, 1QLC [13], and PDZ3, 1BE9 and 1BFE [12]. For human neuronal nitric oxide synthase (nNOS) PDZ, a construct expressing residues 1–132 of nNOS was used (pdb codes: 1QAU [15] and 1B8Q [16]). The typical PDZ domain consists of six β-strands, βA–βF and two α-helices, αA and αB [12–16]. For each of the PDZ domains a Trp residue was introduced as a fluorescent probe [10,17–19] for the folding studies: PDZ1 F95W; PDZ2 Y190W, and PDZ3 F337W. These three Trps were situated in the βC strand of the respective PDZ domain, in the corresponding position to that used before in folding studies of PTP-BL PDZ2 [10,11] and PDZ3 [17]. The corresponding mutant of nNOS PDZ, I42W (numbering according to full-length nNOS, used in Hillier et al. [15]), precipitated easily and

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Abbreviations: PDZ, postsynaptic density protein-95, disc-large tumor suppressor protein, zonula occludens-1; TS, transition state

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PSD-95 PDZ2	A	155
PTP-BL PDZ2	P K P	10
nNOS PDZ	M E D H M F G V Q Q I Q P	13
	* * (positions 15, 16)	
PSD-95 PDZ1	M E Y E E I T L E R G N S G L G F S I A G G T D N P H I G D D P S I **F**	95
PSD-95 PDZ2	E K V M E I K L I K G P K G L G F S I A G G V G N Q H I P G D N S I **Y**	190
PSD-95 PDZ3	R E P R R I V I H R G S T G L G F N I V G G - - - - - - E D G E G I **F**	337
PTP-BL PDZ2	G D T F E V E L A K T D G S L G I S V T G G V - N T S V - R H G G I **Y**	43
nNOS PDZ	N V I S V R L **F** K R K V G G L G F L V K - - - - - - E R V S K P P V I	42
	* * * * * * * (positions 8, 10, 14, 20, 23, 25, 26)	
PSD-95 PDZ1	I T K I I P G G A A A Q D G R L R V N D S I L F V N E V D V R E V T H	130
PSD-95 PDZ2	V T K I I E G G A A H K D G R L Q I G D K I L A V N S V G L E D V M H	225
PSD-95 PDZ3	I S F I L A G G P A D L S G E L R K G D Q I L S V N G V D L R N A S H	372
PTP-BL PDZ2	V K A I I P K G A A E S D G R I H K G D R V L A V N G V S L E G A T H	78
nNOS PDZ	I S D L I R G G A A E Q S G L I Q A G D I I L A V N G R P L V D L S Y	77
	* * * (positions 3, 7, 16)	
PSD-95 PDZ1	S A A V E A L K E - - A G S I V R L Y V M R R	151
PSD-95 PDZ2	E D A V A A L K N - - T Y D V V Y L K V A K P S N A	249
PSD-95 PDZ3	E Q A A I A L K N - - A G Q T V T I I A Q Y K P E E Y S R F E	401
PTP-BL PDZ2	K Q A V E T L R N - - T G Q V V H L L L E K G Q V P	102
nNOS PDZ	D S A L E V L R G I A S E T H V V L I L R G P E G F T T H L E T T F T	112
nNOS PDZ	G D G T P K T I R V T Q P L G P P T K A	132

Fig. 1. Sequence comparison of the five PDZ domains included in the study. All constructs also contained the His tag MHHHHHPRGS at the N-terminus, except PTP-BL PDZ2 which started with MHHHHHHM. The residues in bold were mutated to Trp to obtain a fluorescent label for the folding studies. Identical conserved residues are denoted by a star, ∗. See Section 2 for details on the numbering.

another Trp mutant, F21W, was used in the folding experiments. Also, the nNOS PDZ contained two additional strands in the C-terminus as compared to PDZ1, 2 and 3. The reason was that the shorter nNOS PDZ 1–99 construct expressed poorly and was also precipitating easily.

2.2. Protein purification

The cDNAs encoding PDZ1, PDZ2, PDZ3 and nNOS PDZ were cloned into the BamH1 and EcoR1 restriction sites of a modified pRSET vector (Invitrogen), resulting in clones expressing His-tagged PDZ domain. Protein expression and purification were as described [18,20] but the final purification step was different for PDZ2 and nNOS PDZ after nickel (II)-charged chelating sepharose column chromatography: A concentrated sample of nNOS PDZ was passed through a Q-Sepharose column equilibrated with 50 mM Tris–HCl pH 8.5 and the unbound fraction contained pure nNOS PDZ. For PDZ2 the most concentrated samples after the nickel column were pooled together and loaded on a Superdex-75 (GE healthcare) gel filtration column equilibrated with 20 mM Tris–HCl pH 7.5. Fractions containing pure PDZ as judged from SDS–PAGE were pooled. MALDI-TOF mass spectrometry was used to confirm the identity of the purified PDZ domains and their concentrations were determined from extinction coefficients calculated from amino acid analysis or standard values of the amino acid residues [21].

2.3. Stopped-flow spectroscopy

Kinetic folding and unfolding rate constants were measured on SX17 and SX18 stopped-flow spectrometers from Applied Photophysics (Leatherhead, UK). PDZ domain in buffer with or without urea was mixed to final concentrations of 2–3 μM with buffer-urea solutions to induce either refolding or unfolding of the protein. Excitation was at 280 nm and the change in Trp emission with time was recorded either above 320 nm using a cut-off filter or around 330 nm using an interference emission filter.

2.4. Data analysis

The kinetic data were analyzed assuming a linear dependence of the logarithm of the rate constants on urea concentration [22]. The linear version of the equations are

$$k_f = k_f^{H_2O}\exp(-m_{D\text{-}\ddagger}[\text{urea}]/RT) \quad (1)$$

$$k_u = k_u^{H_2O}\exp(m_{\ddagger\text{-}N}[\text{urea}]/RT) \quad (2)$$

$m_{D\text{-}\ddagger}$ and $m_{\ddagger\text{-}N}$ are the respective slopes of the dependence of log k_f and log k_u on [urea]. The equation describing a two-state mechanism is the sum of Eqs. (1) and (2). When there was a change in rate-limiting step in either the refolding or unfolding arm the data were analyzed according to a three-state model and the sum of Eqs. (2) and (3) (refolding) or Eqs. (1) and (4) (unfolding) were used [23].

$$k_f = k_f^{H_2O}\exp(-m_{D\text{-}\ddagger}[\text{urea}]/RT) \times 1/\{1 + K_p\exp(m_p[\text{urea}]/RT)\} \quad (3)$$

$$k_u = k_u^{H_2O}\exp(m_{\ddagger1\text{-}N}[\text{urea}]/RT) \times 1/\{1 + K_p\exp(m_p[\text{urea}]/RT)\} \quad (4)$$

In Eq. (3), K_p is the equilibrium (or partitioning) constant between the denatured and intermediate populations and m_p the associated m value. In the case of Eq. (4), K_p is the partitioning constant between the two transition states and m_p the associated m value.

Global fit of folding kinetics to common β_T values was performed assuming:

$$\beta_{T1} = m_{D\text{-}\ddagger1}/m_{D\text{-}N} \quad (5)$$

$$\beta_{T2} = m_{D\text{-}\ddagger2}/m_{D\text{-}N} = 1 - (m_{\ddagger1\text{-}N} - m_p)/m_{D\text{-}N} \quad (6)$$

Data were analyzed with Kaleidagraph (Synergy Software) and Prism (GraphPad Software, Inc.).

3. Results

3.1. Stability of PDZ constructs

His-tagged versions of the following PDZ domains were expressed and purified as described in Section 2: PDZ1, PDZ2 and PDZ3 from PSD-95, and nNOS PDZ. All four PDZ domains were folded as judged by far-UV circular dichroism measurements (not shown) and fluorescence-monitored denaturation (Fig. 2). Equilibrium denaturation experiments were performed in 50 mM potassium phosphate pH 7.45 by increas-