The Folding Mechanism of BBL: Plasticity of Transition-State Structure Observed within an Ultrafast Folding Protein Family

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Received 17 February 2009; received in revised form 5 May 2009; accepted 8 May 2009
Available online 13 May 2009

Studies on members of protein families with similar structures but divergent sequences provide insights into the effects of sequence composition on the mechanism of folding. Members of the peripheral subunit-binding domain (PSBD) family fold ultrafast and approach the smallest size for cooperatively folding proteins. Φ-value analysis of the PSBDs E3BD and POB reveals folding via nucleation-condensation through structurally very similar, polarized transition states. Here, we present a Φ-value analysis of the family member BBL and found that it also folds by a nucleation-condensation mechanism. The mean Φ values of BBL, E3BD, and POB were near identical, indicating similar fractions of non-covalent interactions being formed in the transition state. Despite the overall conservation of folding mechanism in this protein family, however, the pattern of Φ values determined for BBL revealed a larger dispersion of the folding nucleus across the entire structure, and the transition state was less polarized. The observed plasticity of transition-state structure can be rationalized by the different helix-forming propensities of PSBD sequences. The very strong helix propensity in the first helix of BBL, relative to E3BD and POB, appears to recruit more structure formation in that helix in the transition state at the expense of weaker interactions in the second helix. Differences in sequence composition can modulate transition-state structure of even the smallest natural protein domains.

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Keywords: peripheral subunit-binding domain family; ultrafast protein folding; temperature-jump fluorescence spectroscopy; Φ-value analysis; transition-state movement

Introduction

As proteins become smaller in size, their structural complexity is reduced and their folding mechanisms appear to be correspondingly simpler. Folding of most small single-domain proteins can be described by a barrier-limited, two-state transition, proceeding from an unstructured, or partially structured, denatured ensemble to the native state without significant population of intermediate states.1–3 The apparent two-state folding scenario is in accord with the presence of both a single major transition state or an ensemble of transition states, reflecting the presence of parallel pathways that cross a common saddle point, on the conformational free-energy surface. Major determinants of the mechanism by which a protein folds are its native-state topology, sequence composition, and the stabilities of its different substructures.4–8

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The Protein Folding Mechanism of BBL

Homologous protein families sharing similar three-dimensional structures but divergent sequences are invaluable for dissecting the roles of specific sequences in a background of common topology in protein folding and stability.6-11 Members of the peripheral subunit-binding domain (PSBD) family are homologous, small (~40 residues), all-helical structural domains12-14 of the ubiquitous, large multi-enzyme 2-oxo-acid dehydrogenase complex that plays critical roles in carbohydrate metabolism.15,16 Small size and ultrafast folding makes PSBDs attractive candidates for combined experimental and theoretical approaches, aiming at atomic-detailed descriptions of folding mechanisms and differences therein. The homologues BBL, E3BD, and POB from mesophilic, thermophilic, and hyperthermophilic bacteria, respectively, share essentially identical native structures and similar sequences.17 How- ever, their stabilities and folding rate constants vary 2- and 8-fold, respectively,17,18 and predicted19 helix-forming propensities for secondary structural elements are markedly different (Fig. 1). The folding mechanism of PSBDs is characterized by barrier-limited, apparent two-state transitions.17,20

Fig. 1. Helix-forming propensities of PSBD family members. Helical propensities of BBL (red), E3BD (blue), and POB (green) sequences were calculated at a residue level using the AGADIR algorithm.19 Regions of secondary structural elements, as determined from the corresponding solution NMR structures, are depicted at the top of the panel (shown from left to right along the sequence from the N- to the C-terminus).

Φ-value analysis for structural characterization of the folding transition state of the family member BBL. Our results show that BBL folds by nucleation-condensation through a compact and structurally diffuse transition state with loosely formed native-like interactions, similar to those observed for E3BD and POB. However, the pattern of Φ values determined for BBL was different from those of its homologues. While folding of E3BD and POB nucleates in helix 2, the folding transition state of BBL appears to be evenly dispersed across the whole structure. The result can be explained by the different predicted helix-forming propensities of PSBDs. The high propensity of BBL to form secondary structure plays critical roles in carbohydrate metabolism.15,16

Results

NMR solution structure of BBL-H142W

Wild-type BBL (which we refer to here as BBL) does not contain any natural fluorophore for ultrafast kinetic experiments. In previous studies, we proposed

<table>
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<th>Structural constraints</th>
<th>Statistics for accepted structures</th>
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<tr>
<td>Intra-residue</td>
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<td>Sequential</td>
<td>Rms deviation (±SD)</td>
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<td>Medium range (2 ≤ i−j ≤ 4)</td>
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<td>Long range (1 ≤ i−j ≤ 4)</td>
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<tr>
<td>Primary structure (Å)</td>
<td>RMSD (±SD)</td>
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<tr>
<td>Bond lengths (Å)</td>
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<tr>
<td>Bond angles (°)</td>
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</tr>
<tr>
<td>Improper angles (°)</td>
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<tr>
<td>Average atomic RMSD from the mean structure (±SD)</td>
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<tr>
<td>Residues 130–168 (N, C, C atoms) (Å)</td>
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<td>Residues 130–168 (all heavy atoms) (Å)</td>
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<td>Structural quality (%)</td>
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<tr>
<td>Residues in most favored region of Ramachandran plot</td>
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<tr>
<td>Residues in additional allowed region of Ramachandran plot</td>
<td></td>
</tr>
<tr>
<td>Residues in disallowed region of Ramachandran plot</td>
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the single-point Trp mutant BBL-H142W as a well-suited fluorescent mimic for wild-type protein because of its identical equilibrium denaturation thermodynamics. Here, we aimed to confirm the suitability of BBL-H142W as “pseudo-wild-type” by determining its NMR solution structure and comparing it with that previously determined for BBL. The structure calculation incorporated the NMR restraints detailed in Table 1 to generate an ensemble of 20 well-defined structures [Protein Data Bank (PDB) code: 2wav; Fig. 2a]. The structures of mutant and wild-type protein superimposed very well (Fig. 2b). The average pairwise Cα RMSD for structured sequence space (residues 130–168) was 1.0 Å. The chain topologies are essentially identical and all secondary and tertiary interaction networks are retained. At the position where the probe was introduced (H142W), we observed only a slight rearrangement of the Trp side chain relative to the His side chain present at the respective position in wild-type protein. There was no noticeable dispersion of W142 rotameric side-chain conformations in the ensemble of NMR structures (Fig. 2b). Tertiary interactions of W142 were well defined, as they are for H142 in wild-type BBL: Interactions of L167 in helix 2 with H142 and W142 in helix 1 of BBL and BBL-H142W, respectively, were retained. The results are in agreement with our previous studies where we found that mutation H142W does not introduce any measurable energetic perturbation in the folding thermodynamics of BBL. Accordingly, BBL-H142W is a suitable “pseudo-wild-type” protein for biophysical studies.

**Design of single-point mutations for Φ-value analysis**

Single-point mutations were introduced into BBL-H142W at selected sites that sampled its entire structured sequence region (residues 130–168). We focused on mutating residues involved in tertiary interactions or those probing the formation of helical secondary structure using alanine to glycine (Ala → Gly) mutations. Ala → Gly mutations probe the structure of the folded and the transition state as they do not affect significantly the free energy of the denatured state. We generally designed conservative nondisruptive deletion mutations, which allow for the most reliable structural interpretation of Φ values. Evolutionary conserved residues within the PSBD family were

**Fig. 2.** NMR solution structure of BBL-H142W (PDB code: 2wav) and comparison with wild-type BBL (PDB code: 1w4h). (a) Ensemble of 20 NMR solution structures determined for BBL-H142W. Shown are the protein backbones of individual structures depicted as ribbons and color-coded from the N-terminus (red) to the C-terminus (blue). For reasons of clarity, the very end of the flexible N-terminus (GSQN) is omitted. (b) Alignment of the BBL-H142W structure closest to the ensemble average (red) with that determined previously for wild-type BBL (blue). Shown is the alignment of structured sequence space (residues 130 to 170). Side chains at the site where the Trp fluorescence probe was introduced (H142W) together with tertiary interactions formed with L167 in helix 2 are detailed. The magnified view shows the ensemble of the 20 side-chain conformations of H142W and L167 determined by NMR.
mutated identically as to those reported previously for E3BD and POB. This allowed direct comparison of $\Phi$ values between homologues. Mutation of charged side chains was avoided because of the long-range nature of Coulombic interactions and the complication of data interpretation in terms of $\Phi$ analysis. Moreover, charge interactions on the protein surface are reduced by Debye–Hückel screening under the high-ionic-strength solvent conditions that arose from using the ionic denaturant guanidinium chloride (GdmCl).

Exceptions from the abovementioned guidelines were mutations V154G and D162N. The former was designed to probe the entropic effect of the bulky, solvent-exposed Val side chain for formation of the hairpin in the loop region connecting helix 1 and helix 2. The latter probes the contribution of the side-chain carboxylate at position 162 that is involved in a complex hydrogen bond network formed by residues in the loop and in the N-terminal end of helix 2 (Fig. 3). Residue D162 is conserved among PSBD family members and its interactions are crucial for stability of E3BD and POB and part of their folding nuclei.

Equilibrium denaturation experiments

All folding experiments were performed using our standard solvent conditions for BBL, namely, pH 7.0 buffers with the ionic strength corrected to 200 mM (Materials and Methods). Under such solvent conditions, BBL was maximally stable and devoid of native-state structural heterogeneities.

Fig. 3. Perspective view on the intricate hydrogen bond network at the N-terminal end of helix 2 of BBL, revealed by NMR spectroscopy (PDB code: 2wav). Possible hydrogen bonds between the side-chain carboxylate of D162, the backbone amide of T159 and T152, respectively, and the hydroxyl side chain of residue T159 are indicated as broken black lines. The T159 side chain can, in turn, form a hydrogen bond with the backbone amide of D162. The side chain D162 and its interactions are conserved in the PSBD family.

Fig. 4. Equilibrium thermal denaturation data of BBL-H142W and mutants thereof acquired using far-UV CD spectroscopy. Denaturation curves recorded from mutants are shown in groups corresponding to the three secondary structural elements inherent in BBL and where mutations were made: helix 1 (upper panel), 310-helix and loop (middle panel), and helix 2 (bottom panel). The color code for individual data sets is shown as inset in each panel. Colored lines are data fits to a model based on a standard thermodynamic equation for a two-state equilibrium.
We investigated the stability of BBL mutants using thermal denaturation monitored by far-UV circular dichroism (CD). Fig. 4 shows thermal denaturation curves obtained from BBL-H142W and single-point mutants thereof. All denaturation curves fitted very well to a thermodynamic equation for a two-state equilibrium (Materials and Methods). The curvature in native-state baselines at low temperatures in data fits of strongly destabilized mutants results from cold denaturation and the relative values of unfolding enthalpy, ΔH_{D-N}, and change in heat capacity between native and denatured state, ΔC_{P,D-N}. Fitted thermodynamic parameters are listed in Table 2. Results from thermal denaturation were used to assess which mutants are amenable to Φ-value analysis. The stability of BBL under optimized solvent conditions is only 2.4 kcal/mol at 298 K,17,18 which is some 1–2 kcal/mol lower than that determined for its homologues E3BD and POB.17 The low stability of BBL at room temperature together with its small m_{D-N} value (m_{D-N}=ΔG_{D-N}/Δ[denaturant]) does not allow stability changes of mutants with ΔΔG_{D-N}~1.0 kcal/mol to be determined accurately by equilibrium chemical denaturation since the slope of the pre-transition baseline is not sufficiently well defined for these mutants.18 Thus, at 298 K, this would narrow the range of measurable stability changes induced by mutation and, ultimately, the accuracy of Φ analysis for BBL. Applying the lower cutoff for free-energy changes of mutants that is generally regarded suitable for Φ analysis in studies using our methodology (ΔΔG_{D-N}>0.5–0.6 kcal/mol) resulted in an experimentally accessible window of 0.5–0.6≤ΔΔG_{D-N}~<1.0 kcal/mol at 298 K, which was too small for Φ analysis. We decided, therefore, to perform experiments at reduced temperature (283 K), where the higher stability allowed us to investigate mutants with ΔΔG_{D-N}~<2.0 kcal/mol.

The stability changes of mutants A130G, A134G, I135V, T152S, H166G, A168G, and A168W were too destabilized for accurate determination of ΔΔG_{D-N} by equilibrium denaturation because of poorly defined pre-transition baselines, but ΔΔG_{D-N} values for L158A, D162N, and

<table>
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<th>Mutant</th>
<th>Tm (K)</th>
<th>ΔH_{D-N} (kcal/mol)</th>
<th>ΔΔG_{D-N} (kcal/mol)</th>
<th>[D]_{DEN} (M)</th>
<th>m_{D-N} (kcal/mol M)</th>
<th>ΔG_{D-N} (kcal/mol)</th>
<th>ΔΔG_{D-N} (kcal/mol)</th>
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<td>327.2±0.2</td>
<td>25±5</td>
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Errors are calculated as described in Materials and Methods.

n.d., data not determined because free-energy changes were outside the suitable data window for accurate determination of ΔΔG_{D-N} values (0.5≤ΔΔG_{D-N}~<2.0 kcal/mol).

* Mutant was too strongly destabilized to yield physically meaningful data fitting results.

** Mutant was designed on the background of the BBL pseudo-wild-type BBL-L131W.
V163A could be estimated from kinetic data as discussed below.

For all other mutants, the abovementioned stability criterion for $\Phi$ analysis held, and equilibrium chemical denaturation of those mutants was measured using far-UV CD spectroscopy at 283 K and GdmCl as denaturant (Fig. 5). Data recorded for all mutants fitted very well to a thermodynamic equation for a two-state equilibrium (Materials and Methods). Fitted thermodynamic parameters are summarized in Table 2. The equilibrium $m_{D-N}$ value remained essentially unchanged upon mutation, reporting on the nondisruptive nature of the deletion mutations made. The mean $m_{D-N}$ value $m_{D-N}=0.70\pm 0.04 \text{ kcal mol}^{-1} \text{ M}^{-1}$ was identical with that determined for the pseudo-wild-type BBL-H142W ($m_{D-N}=0.70\pm 0.03 \text{ kcal mol}^{-1} \text{ M}^{-1}$) and with those previously determined for BBL and BBL-H142W at higher temperature (298 K). This observed conservation of $m_{D-N}$ values strongly supports the absence of ground-state effects upon mutation or changes in temperature. We further found a good agreement of values of $\Delta \Delta G_{D-N}$ measured by thermal and chemical denaturation using far-UV CD spectroscopy (Table 2; Fig. 6). However, experimental errors of thermally derived stability changes and the scatter of data in the correlation plot of thermal versus chemical denaturation data increased significantly beyond $\Delta \Delta G_{D,N}>1.5 \text{ kcal/mol}$ (Fig. 6). Again, this divergence can be attributed to the intrinsically low stability of BBL and the sloping and generally less well-defined pre-transition baselines in thermal denaturation experiments for mutants in this stability range. We characterized a total of 26 mutants, of which 17 were suitable for $\Phi$ analysis.

Kinetic experiments

The folding kinetics of $\Phi$-mutants of BBL-H142W was measured using resistive-heating temperature jump (T-jump) fluorescence spectroscopy. For all mutants investigated, we observed kinetic transients in the microsecond time domain, each of which fitted very well to a single exponential function (Fig. 7). The denaturant dependence of the observed relaxation rate constants, in turn, yielded chevron plots that fitted very well to standard equations for a two-state equilibrium.

**Fig. 5.** Equilibrium chemical denaturation data acquired using far-UV CD spectroscopy for BBL-H142W and selected single-point mutants fulfilling the stability criterion for $\Phi$ analysis. Data recorded are shown in groups corresponding to the three secondary structural elements inherent in BBL and where mutations were made: helix 1 (upper panel), $3_{10}$-helix and loop (middle panel), and helix 2 (bottom panel). The color code for individual data sets is the same as in Fig. 4 and shown as inset in each panel. Colored lines are data fits to a model based on a standard thermodynamic equation for a two-state equilibrium.

**Fig. 6.** Correlation plot of stability changes of BBL upon mutation ($\Delta \Delta G_{D,N}$) determined from chemical and thermal denaturation experiments. The gray line with a slope of 1 is a guide to the eye for a perfect correlation.
The fitted kinetic parameters from chevron analysis are listed in Table 3. There was an excellent agreement between the free energy of unfolding, $\Delta G_{D-N}$, calculated from folding and unfolding rate constants extrapolated to zero denaturant concentration and those extrapolated from equilibrium chemical denaturation using CD spectroscopy (Fig. 9a). The equilibrium $m_{D-N}$ values calculated from kinetic measurements ($m_{D-N} = m_{TS-D} + m_{TS-N}$) were also in excellent agreement with those directly determined from equilibrium chemical denaturation (Fig. 9a), demonstrating the validity of the applied two-state formalism\(^3\) for analysis of protein folding experimental data from BBL.

We compared the contribution of conserved residue side chains to protein stability of PSBD family members. Identical mutations yielded very similar stability changes (all given values in kilocalories per mole) among the homologues BBL, POB\(^2\), and E3BD\(^2\) as observed for mutants L138A ($\Delta \Delta G_{D-N}^{BBL} = 1.60 \pm 0.07$, $\Delta \Delta G_{D-N}^{POB} = 1.73 \pm 0.06$), T159S ($\Delta \Delta G_{D-N}^{BBL} = 1.32 \pm 0.06$, $\Delta \Delta G_{D-N}^{POB} = 1.61 \pm 0.06$), and D162N ($\Delta \Delta G_{D-N}^{BBL} = 2.0 \pm 0.2$, $\Delta \Delta G_{D-N}^{POB} = 2.2 \pm 0.2$, $\Delta \Delta G_{D-N}^{E3BD} = 2.1 \pm 0.1$). Mutation of the aspartic acid side chain D162 to asparagine (D162N), localized in the stability hot spot (Fig. 3), yielded a large and identical destabilization for all three homologues. The mutation T159A in BBL and POB was largely destabilizing, as was the mutation of a conserved, branched aliphatic side chain present in all three homologues at position 158 to alanine. On the other hand, conserved residues in the loop region and at the C-terminal end of helix 2 had varying stability contributions (all given values in kilocalories per mole), as observed for A146G ($\Delta \Delta G_{D-N}^{BBL} = 0.56 \pm 0.04$,

\[\text{Fig. 7. Folding relaxation kinetics of BBL mutants. Kinetic transients recorded from BBL-H142W (black) and representative mutants S132G (blue), V154G (cyan), D162N (green), and L167A (red) at their corresponding midpoint concentration of denaturant at 283 K using resistive-heating T-jump fluorescence spectroscopy. Data sets are shown with a constant offset along the y-axis for reasons of clarity. Gray lines are data fits to a mono-exponential decay function.}\]

\[\text{Fig. 8. Plots of observed relaxation rate constant, } k_{\text{obs}} \text{ versus denaturant concentration (chevron analysis) of BBL-H142W and single-point mutants thereof. Data sets recorded are shown in groups corresponding to the three secondary structural elements inherent in BBL and where mutations were made: helix 1 (upper panel), } \beta_{10}\text{-helix and loop (middle panel), and helix 2 (bottom panel). The color code for individual chevron plots is the same as in Fig. 5 and shown as inset in each panel. Colored lines are data fits to a kinetic model based on standard equations for a barrier-limited, two-state transition.}\]
Table 3. Kinetic parameters for folding/unfolding of BBL-H142W and mutants thereof derived from chevron analysis at 283 K

<table>
<thead>
<tr>
<th>Mutant</th>
<th>( k_f ) (s(^{-1}))</th>
<th>( k_m ) (s(^{-1}))</th>
<th>( m_{TS-D} ) (kcal mol(^{-1}) M(^{-1}))</th>
<th>( m_{TS-N} ) (kcal mol(^{-1}) M(^{-1}))</th>
<th>( \Delta G_{TS-N} ) (kcal mol(^{-1}))</th>
<th>( \Psi_f )</th>
</tr>
</thead>
<tbody>
<tr>
<td>H142W</td>
<td>71,000±6500</td>
<td>770±70</td>
<td>0.56±0.03</td>
<td>0.13±0.02</td>
<td>2.55±0.10</td>
<td>Reference</td>
</tr>
<tr>
<td>L131A</td>
<td>31,000±700</td>
<td>1510±290</td>
<td>0.50±0.02</td>
<td>0.18±0.02</td>
<td>1.70±0.12</td>
<td>0.52±0.08</td>
</tr>
<tr>
<td>L132G</td>
<td>40,400±1500</td>
<td>660±170</td>
<td>0.50±0.02</td>
<td>0.21±0.03</td>
<td>2.31±0.16</td>
<td>0.55±0.13</td>
</tr>
<tr>
<td>H135A</td>
<td>35,100±1100</td>
<td>4310±960</td>
<td>0.46±0.02</td>
<td>0.20±0.04</td>
<td>1.18±0.14</td>
<td>0.32±0.06</td>
</tr>
<tr>
<td>L138A</td>
<td>26,400±1300</td>
<td>5300±1500</td>
<td>0.46±0.06</td>
<td>0.16±0.04</td>
<td>0.90±0.19</td>
<td>0.35±0.05</td>
</tr>
<tr>
<td>A140G</td>
<td>31,500±900</td>
<td>1260±420</td>
<td>0.42±0.03</td>
<td>0.23±0.04</td>
<td>1.81±0.20</td>
<td>0.49±0.08</td>
</tr>
<tr>
<td>H144A</td>
<td>33,200±1200</td>
<td>3970±1780</td>
<td>0.41±0.06</td>
<td>0.18±0.06</td>
<td>1.20±0.27</td>
<td>0.31±0.05</td>
</tr>
<tr>
<td>A146G</td>
<td>46,600±1600</td>
<td>910±130</td>
<td>0.50±0.01</td>
<td>0.20±0.02</td>
<td>2.22±0.10</td>
<td>0.42±0.12</td>
</tr>
<tr>
<td>L148G</td>
<td>35,100±1100</td>
<td>4310±960</td>
<td>0.46±0.02</td>
<td>0.20±0.04</td>
<td>1.18±0.14</td>
<td>0.32±0.06</td>
</tr>
<tr>
<td>H149V</td>
<td>34,200±1600</td>
<td>650±180</td>
<td>0.47±0.03</td>
<td>0.22±0.03</td>
<td>2.24±0.19</td>
<td>0.59±0.12</td>
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<tr>
<td>L152A</td>
<td>22,400±2100</td>
<td>5570±2780</td>
<td>0.48±0.12</td>
<td>0.15±0.08</td>
<td>0.78±0.33</td>
<td>0.39±0.06</td>
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<tr>
<td>V154G</td>
<td>28,000±500</td>
<td>760±110</td>
<td>0.47±0.01</td>
<td>0.24±0.02</td>
<td>2.03±0.09</td>
<td>0.54±0.07</td>
</tr>
<tr>
<td>L158A</td>
<td>13,900±1100</td>
<td>6160±790</td>
<td>0.48±0.05</td>
<td>0.23±0.04</td>
<td>0.46±0.12</td>
<td>0.40±0.05</td>
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<tr>
<td>T159S</td>
<td>28,200±1100</td>
<td>2170±640</td>
<td>0.53±0.05</td>
<td>0.20±0.04</td>
<td>1.43±0.19</td>
<td>0.39±0.06</td>
</tr>
<tr>
<td>D162N</td>
<td>13,100±500</td>
<td>3270±280</td>
<td>0.48±0.05</td>
<td>0.17±0.02</td>
<td>0.79±0.07</td>
<td>0.48±0.05</td>
</tr>
<tr>
<td>V163A</td>
<td>19,900±1000</td>
<td>8600±720</td>
<td>0.48±0.05</td>
<td>0.16±0.02</td>
<td>0.47±0.08</td>
<td>0.31±0.04</td>
</tr>
<tr>
<td>H166G</td>
<td>31,300±1700</td>
<td>6050±1830</td>
<td>0.45±0.05</td>
<td>0.21±0.05</td>
<td>0.93±0.20</td>
<td>0.25±0.05</td>
</tr>
<tr>
<td>L167A</td>
<td>33,600±800</td>
<td>770±110</td>
<td>0.54±0.01</td>
<td>0.16±0.02</td>
<td>2.13±0.09</td>
<td>0.71±0.12</td>
</tr>
<tr>
<td>L131W</td>
<td>32,800±2100</td>
<td>640±180</td>
<td>0.52±0.03</td>
<td>0.17±0.03</td>
<td>2.22±0.20</td>
<td>Reference</td>
</tr>
<tr>
<td>L167A*</td>
<td>14,700±700</td>
<td>1100±280</td>
<td>0.55±0.05</td>
<td>0.14±0.03</td>
<td>1.46±0.17</td>
<td>0.61±0.18</td>
</tr>
</tbody>
</table>

Errors reported for BBL-H142W are standard deviations from three measurements, which were similar to the corresponding standard errors of the fit. Errors of all mutants reported are, therefore, given as standard errors from data fits.

* Mutant was designed on the background of the BBL pseudo-wild-type BBL-L131W.

\[ \Delta G_{POB} = 1.52 \pm 0.06 \] and L167A \( \Delta G_{BBL} = 0.60 \pm 0.04 \), \( \Delta G_{PSBD} = 1.3 \pm 0.1 \).

We characterized the relative compactness of the folding transition state of BBL by calculating the Tanford \( \beta \)-value \(^{32} [\beta_T = m_{TS-D} / (m_{TS-D} + m_{TS-N})]\), which represents a measure of the solvent-accessible surface area of the transition state (TS) on a relative scale between denatured (D) and native (N) states. The mean \( \beta_T \) value of BBL mutants investigated in this study was \( \beta_T = 0.71 \pm 0.05 \), a value similar to that previously determined for BBL at higher temperature\(^{18} \) and other PSBD homologues.\(^{17} \) The finding is consistent with the picture of BBL having a compact and native-like transition state for folding that is relatively insensitive to temperature and mutation. A large \( \beta_T \) value, on the other hand, reflects a large sensitivity of the refolding rate constant on denaturant concentration, compared to the unfolding rate constant, giving rise to the observed asymmetry in chevron plots (Fig. 8). Consequently, the refolding arms in chevron plots were much better defined than the unfolding arms. As pointed out above, we were able to measure relaxation rate constants down to low denaturant concentration, often even directly in the absence of denaturant. The refolding rate constants in the absence of denaturant could, therefore, be determined accurately with minimal errors from data extrapolation and contributions from the less well defined, extrapolated unfolding rate constants. Hence, we decided to determine \( \Phi \) values for refolding (\( \Phi_f \)) in the absence of denaturant.

**Φ-Value analysis**

Φ analysis reveals a structural picture of the folding transition state by successively deleting interactions present in the native state, best practiced by using site-directed mutagenesis.\(^{21,22} \) The structure of the folding transition state is inferred from transition-state and ground-state free-energy changes of single-point mutants compared to wild-type protein, measured using kinetic and equilibrium experiments. The \( \Phi_f \) value is defined as \( \Phi_f = - \Delta G_{TS-D} / \Delta G_{TS-N} \). The change of free energy upon mutation for moving from the denatured to the transition state (\( \Delta G_{TS-D} \)), measured by kinetics, is related to the change of free energy upon mutation between denatured and native state, measured by equilibrium experiments. A \( \Phi_f \) value of 1 means that the interaction at the mutational site is present in the transition state as it is in the native state. A \( \Phi_f \) value of 0 means that the interaction in the transition state is denatured-like.\(^{33} \) Fractional \( \Phi_f \) values are more complicated to interpret. For the mutation of larger to smaller hydrophobic side chains, and especially for Ala→Gly mutations, the fraction is a measure of the extent to which native-like interactions are formed in the transition state at the particular site of mutation.\(^ {22,26,29} \)

The \( \Phi_f \) values determined for BBL are shown in Fig. 9b, together with those previously reported for the homologues E3BD and POB determined under similar solvent conditions.\(^{24,25} \) \( \Phi_f \) values of BBL are fractional along the entire sequence, similar to its homologues, and the folding mechanism can be described by nucleation–condensation.\(^{1} \) However, the distribution of the \( \Phi_f \)-value pattern of BBL along the sequence was different from that of E3BD and POB. While the sequence was different from that of E3BD and POB, the distribution of the \( \Phi_f \)-value pattern for BBL was shown to be different from that of E3BD and POB. The result can be seen more clearly by classifying \( \Phi_f \) values as “low” \((0.0 < \Phi_f \leq 0.3)\), “medium” \((0.3 < \Phi_f \leq 0.6)\), and
(0.6 < \Phi_f \leq 1.0) and mapping them onto the corresponding native-state structure (Fig. 9c). While folding of E3BD and POB nucleates in helix 2, the folding nucleus of BBL appears to be dispersed across the whole structure. Mutation of the solvent-exposed alanine residues A140 and A148 in helix 1 and in the 310-helical turn of BBL, respectively, to glycine is a good probe for involvement of helical secondary structure in the folding transition state.26 \Phi_f values of 0.49±0.08 and
Discussion

Members of the PSBD family approach the size of the smallest, naturally occurring protein domains. Ultrafast folding and the high degree of structural similarity make them an interesting system to investigate the effect of sequence changes on the folding mechanism of small, single-domain proteins. The native-state structures of the three homologues have topologically identical interaction networks composed of very similar or even identical amino acid side chains. This is reflected in results comparing free-energy changes of unfolding of similar or identical side-chain mutations between family members. The similarities in the large reduction of protein stability upon mutation of certain residues that are conserved within the family are striking. The region most sensitive to mutation involves an irregular loop at the N-terminal end of the second helix and an intricate, buried hydrogen bond network with side-chain interactions involved (Fig. 3). The carboxylic acid group of the D162 side chain in this network loses an identical stability increment of \( \Delta \Delta G_{\text{D,N}} = 2.1 \pm 0.1 \) kcal/mol for all three family members. Destabilization upon removal of the aliphatic side chain at position 158 is similarly large. Similarities of native-state topologies and critical interaction networks of PSBDs extend to their folding mechanisms.

All three homologues fold on microsecond time scales via barrier-limited, apparent two-state transitions. The irregular loop, as well as the associated sensitive hydrogen bond network at the N-terminal end of the second helix, is the topologically favorable nucleation site for folding since it allows many native interactions in short sequence distance. Indeed, \( \Phi \) analyses of E3BD and POB reveal similarly polarized transition states, with the folding nucleus being located in the second helix coinciding with the location of the stability hot spot (Fig. 9b and c). The similarity of high \( \Phi \) values in the second helix of E3BD and POB that contrasts differences in their predicted helical propensities in helix 2 can be explained by the sequence area being shared with the folding nucleus. This interpretation is supported by the comparison of the rank order of folding rate constants with predicted helix-forming propensities in this region. The rank order of folding rate constants determined for E3BD, BBL, and POB at 298 K of \( k_f = 27,500 \pm 500 \) s\(^{-1}\) \( \pm 1,17 \) \( \pm 1,18 \) \( \pm 210,000 \pm 5000 \) s\(^{-1}\), respectively, follows the rank order of predicted helical propen-
sities in the second helix (Fig. 1). An increased helical propensity at the nucleation site appears to stabilize the folding nucleus and results in an increased folding rate constant.

In the present study, Φ analysis of BBL yielded fractional Φ values as has been observed for E3BD and POB. The observed patterns are characteristic for a nucleation-condensation mechanism that describes a coupled process in which the formation of the nucleus and the formation of structure elsewhere are concerted. All three homologues have similarly high βT values, reporting on a compact and native-like transition-state structure. Moreover, the mean Φ values averaged along the respective sequence of family members are in excellent agreement (ΦE3BD = 0.44 ± 0.12, ΦPOD = 0.44 ± 0.21, and ΦE3BD = 0.43 ± 0.21). The pattern of individual Φ values determined for BBL, however, is significantly different from that of its homologues. Φ values of BBL were predominantly “medium” (0.3–0.5) and distributed evenly along the entire sequence (Fig. 9b and c). The folding nucleus in the second helix of BBL appears to be dispersed into the first one. The observation can be explained by the higher intrinsic helix-forming propensity of BBL compared to E3BD and POB in the sequence area of helix 1 (Fig. 1). The high helical propensity of BBL here leads to early recruitment of N-terminal interaction networks during folding at the expense of C-terminal interactions, and the folding transition state of BBL appears to be structured in helix 1 and less structured in helix 2 compared to E3BD and POB. There appears to be a balance between the topologically favorable nucleation site and the local propensity to form secondary structure dictating the pattern of interaction networks present in the transition state for folding of PSBDs. It is worth mentioning that the involvement of side-chain interactions that are distributed along the entire sequence area in the folding transition state, as observed here for BBL, underscores the cooperative nature of folding.

In previous studies on larger proteins, it has been shown that the transition state can move on the reaction free-energy surface according to the same principles as found in classical chemical mechanisms for covalent bond formation. For example, movement along the reaction coordinate has been found according to the Hammond postulate. Movement perpendicular to the reaction coordinate, corresponding to anti-Hammond behavior, has also been observed. The differences in Φ-value pattern observed within the PSBD family lie in with the concept of a malleable folding pathway. It has been suggested that the number of folding pathways of a protein is determined by the number of nucleation-competent submotifs, so-called “foldons”, and how they are coupled. Following this argument, ideal two-state folders contain one foldon, and the pathway by which they fold is robust. If proteins contain multiple, overlapping foldons, the folding progression can be broad and partitioned over multiple pathways. The dispersion of a folding nucleus induced by sequence variation, as observed here, reflects a transverse transition-state shift across the folding energy landscape. In general, such transition-state shifts are characterized by decreased Φ values in one part of the structure, balanced by increased Φ values in another. Exactly this type of behavior is observed in BBL that exhibits increased Φ values in helix 1 and decreased ones in the helix 2 compared with E3BD and POB, but the total amount of interactions formed in the transition state is retained.

Pathway malleability was originally proposed for proteins that can be decomposed into independent, cooperatively folding subunits. Indeed, Φ analysis performed on such proteins reveals that structurally related ones can, in some cases, have variation of transition-state structure. Members of the ultrafast folding PSBD family, though, belong to the class of the smallest, cooperatively folding protein domains known to date and approach the size of a foldon. Results from the present study suggest that even the smallest globular protein domains with conserved native structure and overall folding mechanism can exhibit plasticity of transition-state structure.

Materials and Methods

Protein expression and sample preparation

BBL mutants were generated using a Stratagene Quick-Change mutagenesis kit. Recombinant mutants were over-expressed in Escherichia coli C41 (DE3) as His-tagged C-terminal fusion proteins in modified pRSETA (Invitrogen) and purified as described elsewhere. In brevity, fusion proteins were isolated from cell lysate by affinity chromatography and BBL variants were cleaved from the fusion protein by site-directed proteolysis. BBL mutants were further purified to homogeneity using ion-exchange and size-exclusion chromatography. Pooled protein fractions were lyophilised. Protein purity was checked using reversed-phase HPLC and protein identity was confirmed using matrix-assisted laser desorption mass spectrometry.

All reagents were purchased from Sigma-Aldrich, with the exception of ultrapure GdmCl, which was purchased from ICN Biomedicals. GdmCl concentrations for stock solutions were assayed by refractometry. Thermal denaturation experiments were performed using 50 mM potassium phosphate, pH 7.0, with the ionic strength adjusted to 200 mM using potassium chloride. Chemical denaturation experiments were performed in 50 mM 3-[morpholino]propanesulfonic acid, pH 7.0, with the ionic strength adjusted to 200 mM using sodium chloride. All sample solutions were filtered using 0.2-μm syringe filters before measurement. Samples for T-jump fluorescence spectroscopy were additionally degassed in vacuum prior to kinetic experiments.

NMR spectroscopy

All NMR spectra were recorded on Bruker Advance-800, Advance-600, and DMX-500 spectrometers. 2D nuclear Overhauser enhancement (NOE) spectroscopy (150 ms mixing time), total correlated spectroscopy, double quantum filtered correlated spectroscopy, and 15N-heteronuclear single quantum coherence and 3D
HNCACB, CBCACONH, HNCO, and HNCA CO spectra of unlabelled or uniformly $^{13}$N-$^{15}$C-labeled BBL-H142W were recorded at 298 K with $-600 \mu\text{m}$ protein in 50 mM potassium phosphate, pH 7.0, containing 5% D$_2$O with the ionic strength adjusted to 200 mM using potassium chloride. Proton chemical shifts were referenced relative to external sodium 2,2-dimethyl-2-silapentane-5-sulfonate, and carbon and nitrogen chemical shifts were re-

ferred indirectly using relative frequencies as described previously.$^{43}$

Spectra were processed with TopSpin (Bruker) and analyzed with Sparky 3.1 (T. D. Goddard and D. G. Kneller, SPARKY 3, University of California, San Francisco) and ANSIG v3.3.$^{44}$ Backbone and some side-chain resonance assignments were made by standard triple-

resonance procedures;$^{45}$ further side-chain resonances assignments were made by standard 2D procedures.$^{46}$ Inter-proton distance constraints were derived from the integrals of cross peaks in the homonuclear NOE spectroscopy spectrum, classified into four categories (1.8–2.8, 1.8–3.5, 1.8–4.75, and 1.8–6.0 A), calibrated by reference to standard inter-residue distances within an $\alpha$-helix. Backbone $\psi$ and $\psi$ torsion angle constraints were obtained from chemical shifts using the program TALOS,$^{47}$ and side-chain $\chi$' constraints were derived from inspections of NOE intensity during stereospecific assignment of pro-

chiral protons. Hydrogen bond donor amides were identified as slow exchanging in heteronuclear single quantum coherence spectra obtained (at 283 K in order to lower the exchange rate, which is very high at pH 7) after dissolving lyophilized protein in NMR buffer containing 99.9% deuterium oxide. Their cognate acceptors were identified in the latter stages of structure determination by analyzing the structural ensemble with the program hplus.$^{48}$ Hydrogen bond constraints of 1.8–2.1 A were imposed on the distance between the hydrogen and the acceptor oxygen, while another constraint of 2.7–3.1 A was imposed on the distance between the donor nitrogen and the acceptor oxygen.

Structures were calculated using a standard torsion angle dynamics simulated annealing protocol in the program CNS.$^{49}$ The input to iterative rounds of structure calculations comprised, in order of inclusion, NOE intensity-derived distance constraints in four categories (corresponding to inter-proton distance constraints of 1.8–2.8, 1.8–3.5, 1.8–4.75, and 1.8–6.0 A), backbone $\psi$ and $\psi$ torsion angle constraints, side-chain torsion angle, and fixed hydrogen bond distance constraints. Twenty structures were accepted where no distance violations were greater than 0.25 A and no angle violations were greater than 5.0°. The constraints and results of the structure calculations are summarized in Table 1.

Graphical images of molecular structures were generated using the software tool VMD$^{50}$ and rendered using the software POVRay.

**Far-UV CD**

Thermal and chemical equilibrium denaturation experiments were performed by far-UV CD spectroscopy using a Jasco J815 spectropolarimeter. Thermal denaturation was monitored in the far-UV CD absorption band for helical secondary structure at 214 nm instead of 222 nm (the absorption maximum) in order to minimize the wave-

length-dependent contributions of the aromatic Trp residue to the far-UV CD signal, which can give rise to steep pre-transition baselines.$^{18}$ A 1-mm path-length cell (Hellma) and protein concentrations of 50 $\mu$M were used throughout all experiments. Temperature was ramped from 275 to 371 K for thermal denaturation using a Peltier temperature controller. Chemical denaturation was performed by manual titration between 0 and 8 M GdmCl (0.2-M increments) at 283 K. In chemical denaturation experiments, baselines were found to be non-sloping and the CD signal was monitored at the wavelength of maximal ellipticity (222 nm).

**T-jump fluorescence spectroscopy**

Relaxation kinetics was measured using resistive-heating T-jump fluorescence spectroscopy. T-jumps of 3 K, from 280 to 283 K, were induced using a modified Hi-Tech PTJ-64 capacitor-discharge apparatus, equipped with a 30-nF capacitor and a 5 mm $\times$ 5 mm measurement cell resulting in an instrumental heating time of $\sim$20 $\mu$s. The sample concentration was typically 50 $\mu$M protein. At the edges of the folding/unfolding transition of the folding equilibrium where the amplitudes of kinetics were reduced due to small changes in population upon perturbation, increased protein concentrations (of up to 100 $\mu$M) were used to improve the signal-to-noise ratio of recorded transients. Trp fluorescence was excited at 280 nm using a mercury–xenon lamp and an optical high-transmittance band-pass filter. Fluorescence emission at $\sim$330 nm was collected using an optical cutoff filter. Between 10 and 20 shots were averaged, as needed, to obtain acceptable signal-to-noise ratio of transients.

**Data analysis of protein folding experiments**

Equilibrium protein denaturation data were fitted using models based on the classical thermodynamic equations for a two-state equilibrium between native and denatured conformational states, as detailed elsewhere.$^{19}$

Thermal denaturation data were fitted using a model based on the equation for the temperature dependence of the free energy of a two-state equilibrium between native (N) and denatured (D) protein:

$$
\Delta G_{D-N}(T) = \Delta H_{D-N}^{m} \cdot \left(1 - \frac{T}{T_{m}}\right) - \Delta C_{p,D-N} \cdot \left(T_{m} - T + \ln \left(\frac{T}{T_{m}}\right)\right)
$$

(1)

where $\Delta H_{D-N}^{m}$ is the enthalpy of unfolding at the transition midpoint, $T_{m}$ is the midpoint temperature, and $\Delta C_{p,D-N}$ is the difference in heat capacity between native and denatured state. From our previous experimental studies on the PSBD family,$^{17}$ we estimated $\Delta C_{p,D,N}$ of BBL to 350±40 cal mol$^{-1}$ K$^{-1}$. Free-energy changes between BBL-H142W and mutant protein ($\Delta \Delta G_{D-N}$) were calculated using the Schellman formalism$^{33}$ assuming conservation of midpoint entropies for nondisruptive deletion mutations:

$$
\Delta \Delta G_{D-N} = \Delta S_{D,N}^{m} \cdot \Delta T_{m} = \frac{\Delta H_{D,N}^{m}}{T_{m}} \cdot \Delta T_{m}
$$

(2)

where $\Delta S_{D,N}^{m}$ is the midpoint entropy ($\Delta H_{D,N}^{m}/T_{m}$) of wild-

type protein and $\Delta T_{m}$ is the shift in midpoint temperature between wild-type and mutant protein. Experimental errors for $\Delta H_{D,N}^{m}$ and $\Delta C_{p,D-N}$ were estimated as described previously$^{32}$ and propagated to yield errors for $\Delta \Delta G_{D-N}$.

Chemical denaturation data were fitted using a model based on the classical, linear free-energy relationship:$^{53}$

$$
\Delta G_{D-N} = \langle[D]\rangle = \Delta G_{D-N} - m_{D,N} \cdot \langle[D]\rangle
$$

(3)
where \([D]\) is the concentration of denaturant (GdmCl) and \(m_{D-N}\) is the equilibrium \(n\) value. Experimental errors for \(\Delta\Delta_{D-N}\) and \(m_{D-N}\) were estimated from standard errors of data fits. Nondisruptive deletion mutations, as employed here, do generally not lead to significant changes in ground states, reflected in constant \(m_{D-N}\) values observed in equilibrium denaturation experiments. \(\Delta\Delta_{D-N}\) Values in standard solvent conditions can therefore be calculated using the relation:

\[
\Delta\Delta_{D-N} = m_{D-N} \cdot \Delta[D]_{50%} \tag{4}
\]

where \([D]_{50%}\) is the concentration of GdmCl at the transition midpoint and \(m_{D-N}\) represents the averaged equilibrium \(m_{D-N}\) value (\(m_{D-N}=0.72\pm0.02 \text{ kcal mol}^{-1} \text{M}^{-1}\)) determined from mutants where individual \(m_{D-N}\) values could be determined reliably (mutants with \([D]_{50%} \geq 2 \text{ M GdmCl}\)).

All kinetic transients recorded fitted very well to single-exponential decay functions. Chevron analysis follows the linear free-energy relationship for chemical perturbation of a barrier-limited, two-state equilibrium, and the observed relaxation rate constant for such a system can be expressed as a function of denaturant concentration:

\[
\log k_{obs}([D]) = \log[k_{f}exp(−m_{TS-D}[D]/RT) + k_{f}exp(m_{TS-N}[D]/RT)] - k_{f}T
\]

where \(m_{TS-D}\) and \(m_{TS-N}\) are the kinetic folding and unfolding \(n\) values, respectively; \(k_{f}\) and \(k_{u}\) are microscopic folding and unfolding rate constants under standard solvent conditions, respectively; \(R\) is the gas constant; and \(T\) is the experimental temperature. All chevron data were fitted to Eq. (5). Reported errors are standard errors from data fits.

In \(\Phi_{f}\)-value analysis, the difference of the height of the free-energy barrier between wild-type and mutant going from the denatured (D) to the transition state (TS), \(\Delta\Delta_{TS-D}\), is divided to the free-energy difference of the corresponding ground states (\(\Delta\Delta_{D-N}\)):

\[
\Phi_{f} = \frac{-\Delta\Delta_{TS-D}}{\Delta\Delta_{D-N}} \tag{6}
\]

\(\Delta\Delta_{TS-D}\) can be determined from the folding rate constants of wild-type (\(k_{f}^{W}\)) and mutant (\(k_{f}^{M}\)) protein:

\[
\Delta\Delta_{TS-D} = RT\ln\left(\frac{k_{f}^{W}}{k_{f}^{M}}\right) \tag{7}
\]

Errors of \(\Phi_{f}\) were determined from propagated errors of folding rate constants and \(\Delta\Delta_{D-N}\) values.

Data deposition

The atomic coordinates for BBL-H142W have been deposited in the PDB (access code: 2wav).

Acknowledgement

Hannes Neuweiler was supported by a Marie Curie Intra-European Fellowship (6th Framework Program of the European Union).

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