

The sixth Datta Lecture

Protein folding and stability: the pathway of folding of barnase

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The pathway of folding of a protein will be completely solved when the structures and energetics of the initial unfolded states, all folding intermediates, all transition states and the final folded state, have been determined. The ultimate goal is to analyse, at the detail of individual residues, the non-covalent interactions that are primarily responsible for dictating secondary and tertiary structure. Until recently, the tools for tackling such a daunting task were quite inadequate, but recent developments in NMR and protein engineering have made it possible to determine crucial features in the folding process. It now seems feasible that sufficient experimental detail will be obtained to provide general principles that govern protein folding and provide the basis for its rigorous theoretical analysis. This lecture will outline the progress and prospects in attainment of the goals as applied to the small ribonuclease, barnase.

Protein engineering; Protein folding; Protein stability; Barnase

1. INTRODUCTION

Most proteins refold spontaneously when their denatured states are put under conditions that favour the folded state. The amino acid sequences of those proteins, therefore, contain the information that directs their three-dimensional structure. The elucidation of the rules that govern folding is a great unsolved problem, for it is not yet possible to calculate *de novo* the pathway of folding of a protein or even whether a known structure is stable. Levinthal [1] pointed out that there are so many conformations available to an unfolded polypeptide chain that the age of the universe would be too short for all the available conformations to be explored by rotating the backbone Φ - and Ψ -angles at random. Consequently, there must be co-ordinated movements of amino acid residues during folding [2]. Further, the observed free energies of folding of proteins are very small, at some 5–15 kcal·mol⁻¹ for a typical protein of 100–200 residues. Those small numbers are the differences between two large numbers, the free energies of the non-covalent interactions in the folded and unfolded states, neither of which can be calculated

with sufficient precision given the uncertainties in the methods currently used for interactions in solution [3,4]. The rational design of novel proteins requires both a detailed prediction of the energetics of folding and an understanding of the pathway since there could be essential initiation sites or even kinetic blocks to folding. Some proteins, for example, require chaperones to prevent aggregation occurring in competition with folding [5], and others require propeptide sequences to overcome kinetic blocks [6]. Experimental evidence is thus required about the energetics of interactions within proteins, and simplifying factors during the pathway of folding. Questions that are asked are: what is the order of events during folding? Are there initiation sites? Do segments of structure form independently? Are there stop and start signals for elements of structure? Are there parallel pathways of folding? What drives structure formation? General principles may not be sufficient in themselves to predict the folding of specific molecules because the energy balance between different folded states could depend on specific local interactions. Earlier work on protein folding has been extensively reviewed [7–9].

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2. THE TARGETS AND THE METHODS

The different states in a folding pathway and the methods available for examining them are illustrated in Fig. 1. The key goal is to analyse the non-covalent interactions at the level of individual residues. Stable intermediates may be studied by general spectroscopic

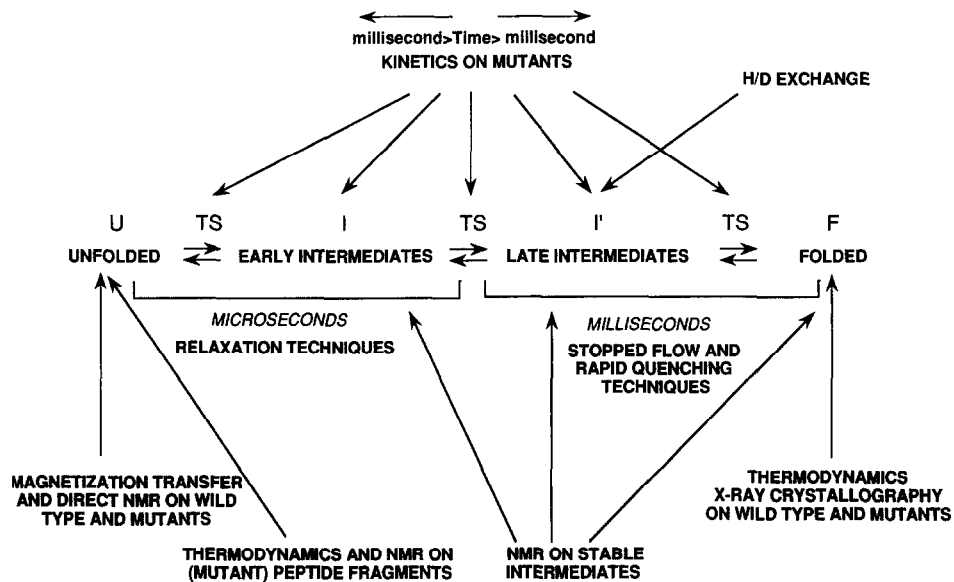


Fig. 1. Strategy for analysing the pathway of protein folding and the analytical methods available.

methods whereas the properties of transition states and transient intermediates must be inferred from kinetics. Stable intermediates may be side products, and so their kinetic properties must be examined to see if they are on the reaction pathway. The characterisation of folding pathways thus requires a combination of kinetic and structural analysis. Neither kinetics nor structure by themselves are sufficient for describing pathways. The time scale of kinetics may be divided into two regimes; greater or less than 1 ms. The physical mixing of two solutions takes a fraction of a millisecond, and so techniques such as stopped-flow or rapid mixing and sampling are restricted to time scales of 1 ms or greater. The monitoring of faster events requires that the solutions are already mixed. Relaxation methods, such as temperature-jump ($t_{1/2} > 1 \mu\text{s}$) or pressure-jump ($t_{1/2} > 50 \mu\text{s}$) kinetics must be employed, or initiation of a reaction by flash photolysis could be used.

Earlier kinetic studies tended to use global probes of structure to monitor overall folding or the formation of domains, such as changes in tryptophan fluorescence or absorbance. Increasing sophistication has led to the introduction of stopped-flow circular dichroism (CD) to monitor the formation of secondary structure [10]. This is similarly limited to being an overall probe of secondary structure. The major CD signal is for helix formation from ellipticity at 222 nm. Detail is required at even higher resolution, however. A combination of quenched-flow and NMR measurements of backbone NH H/D-exchange has been able to monitor the formation of secondary structure at some individual positions in suitable proteins (see below [11–14]). Protein engineering is another candidate for detailed analysis [15,16]. An experimental approach, based on protein engineering, had been introduced in work on the ty-

rosyl-tRNA synthetase [17] to explore the roles of interaction energies between substrates and the individual side chains of an enzyme during catalysis [18,19]. This procedure is directly applicable to mapping out in fine detail the extent of structure formation and the energetics at almost every side chain in a protein during unfolding and refolding [20,21]. The development of the theory of the protein engineering method and its successful application to the folding of a particular protein, barnase, has recently been published in detail [22–27]. My laboratory is now using an integrated experimental approach to protein folding that employs a range of biophysical methods applied to protein-engineered mutants and their peptide fragments. The work hinges on protein engineering as it is essential for making mutants that have suitable properties and modifications for study by biophysical methods. Further, it is possible to obtain elusive information in fine detail by measuring the thermodynamic and kinetic properties of engineered mutants per se.

3. THE PROTEIN ENGINEERING STRATEGY

3.1. The ϕ -value procedure

We are using a two-part strategy for measuring the energetics of non-covalent interactions within proteins and for probing the pathway of folding. Site-directed mutagenesis is used to remove parts of side chains the interactions of which stabilise regions of the protein. The first part of the strategy is to measure the changes in stability of the protein on mutation in order to establish an empirical thermodynamic data base for use when redesigning enzymes or for testing theoretical procedures. These results have recently been reviewed [28]. The second part is to make kinetic measurements on

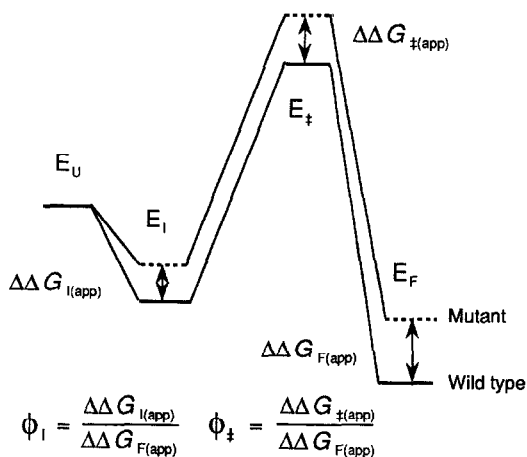


Fig. 2. Partial free energy diagrams for the folding of wild-type and mutant enzymes. E_U , E_I , E_{\ddagger} and E_F are the unfolded, intermediate, transition and folded states, respectively. The free energy levels of the unfolded states of both enzymes are shown to be the same for illustrative convenience. It has been demonstrated, as an essential development of the underlying theory, that the values of ϕ are independent of this [21,22]. This is born out in practice on comparing the mutation of buried valine residues to both Ala and Thr [24,25]: (Val→Ala disturbs primarily the folded state, Val→Thr lowers the energy of the unfolded state [22].)

unfolding and refolding of mutant enzymes to measure the changes in activation energies and the energy levels of any intermediates (Fig. 2). The changes in energy levels may be used to map the structures of transition states and intermediates. Each mutation acts as a reporter group for the events that occur at the target region during folding.

There are changes of the energy levels of the folding intermediates and transition states on mutation. These changes are divided by the change in the overall free energy of folding to give a series of values of a parameter, ϕ (Fig. 2). ϕ is similar to but differs from the Brønsted β -value of physical organic chemistry [20,22]. ϕ and β are the same, however, at the two extreme values of 0 and 1, which are often found in practice and have a simple interpretation. A value of $\phi = 0$ (measured from the unfolded state and in the direction of folding) implies that the structure at the site of mutation is as unfolded in the transition state or intermediate as it is in the unfolded state. Conversely, $\phi = 1$ shows that the structure at the site of mutation is as folded in the transition state as it is in the folded structure. Fractional values of ϕ are more difficult to interpret since they may imply that there is either partial non-covalent bond formation or a mixture of states. The major assumptions of the method are: (i) the mutation does not alter the pathway of folding; (ii) the mutation does not significantly change the structure of the folded state; (iii) the mutation does not perturb the structure of the unfolded state; and (iv) the target groups do not make new interactions with new partners during the reaction. Assump-

tions (ii) and (iii) are not necessarily essential for the simple cases of $\phi = 0$ or 1, the most common values, since effects of disruption of structure can cancel out. Assumption (iv) may be directly tested by the double-mutant technique, described below (section 3.3.) since both residues that make a particular interaction may be mutated [29,30].

3.2. Choice of mutation

The high resolution structure of the protein is examined for interactions that provide probes of the integrity within a particular substructure or the interaction between elements of structure. The ideal mutations are those that delete small parts of side chains that make simple and defined interactions and do not cause any disruption of structure other than removal of those interactions. We have termed such ideal mutations as 'non-disruptive deletions' [31]. The smaller the mutation the better since the smaller the perturbation of the system. When in doubt, we mutate to Ala since this will avoid steric clashes and does not introduce a group that could form adventitious hydrogen bonds. We avoid mutations that leave buried charged groups unpaired since they are so unstable that the structure will inevitably rearrange to allow solvation. Mutation of a buried side chain to a that of a larger residue or one of different branching will most likely lead to complications in analysis because of steric repulsion. Sensibly designed mutations do often correspond well to being non-disruptive deletions (see section 3.3.).

The fine structure of interactions may sometimes be analysed by a series of mutations at a single site: e.g. Ile→Val gives the interactions of the δ -methyl group, and the further mutation of Val→Ala gives those of the γ -methyls [22]. Mutation of buried valines to both Ala and Thr gives complementary information since the deletion to Ala alters stability primarily by destabilising the folded state, whereas the isosteric switch of a $-CH_3$ to an $-OH$ destabilises a protein by stabilising the unfolded state.

3.3. The double-mutant cycle method

A further refinement is the use of the double-mutant cycle technique [32]. The larger side chains can make a multitude of interactions within a protein. Particular interactions between two residues in the protein may, however, frequently be separated from the rest as follows [33]. Two residues, X and Y, in a protein E that interact are mutated separately and then together to give a cycle comprising E–XY, EX, EY and E. In ideal circumstances, where the mutations cause no rearrangement in the protein structure, subtraction of the free energy change $\Delta G_{E-X \rightarrow E}$ (for the process of E–X→E) from $\Delta G_{E-XY \rightarrow EY}$ (the free energy change for E–XY→EY) leads to the interaction energies of X and Y with the rest of the protein to cancel out [33,34]. This still holds if there is disruption of the structure of the

enzyme on mutation of X in E–XY but the same disruption occurs on the mutation of X in E–X. Structural artefacts on mutation thus tend to cancel each other out, reducing their importance to second order effects.

We now discuss how the methods have been applied to barnase, working backwards from the folded structure.

4. THE FOLDED STATE OF BARNASE

Barnase is a monomeric, single chain, RNase of 110 residues and M_r 12,382 that is secreted by *Bacillus amyloliquefaciens* (Fig. 3). It has been cloned and over-expressed in *Escherichia coli* [35], its crystal structure determined at high resolution ([36,37] and A. Cameron (York University) and Kim Henrick, unpublished), and its solution structure determined by NMR [38]. Barnase provides a particularly good paradigm for studying folding: (i) it undergoes reversible unfolding, induced by heat, urea or guanidinium chloride, which fits to a two-state process at equilibrium; (ii) there are no disulphide linkages to constrain the unfolded state – it unfolds more completely than any other protein yet studied, and refolding can be initiated from the fully unfolded state; (iii) its three prolines are all *trans* in the folded state and so the kinetics of refolding are largely unaffected by the rate of *cis*–*trans* isomerisation of the small fraction of prolines that equilibrate to *cis* in the unfolded state [39]; and (iv) the protein is an α + β protein in which the α and β elements of secondary structure are in different parts of the protein and so may be separated in studies on protein fragments.

Barnase has a major α -helix (helix₁) spanning residues 6–18. The first and last residues, the caps (Thr-6 and His-18), have side chains that form hydrogen bonds with the exposed NH and CO groups at the N- and C-termini of the helix. A second α -helix (helix₂) spans residues 26–34 and has also a Thr (Thr-26) as the N-cap. There is also α -helix₃, which consists of just one turn, residues 41–46. There are five strands of β -sheet (β_1 , residues 50–55; β_2 , 70–76; β_3 , 85–91; β_4 , 94–99; and β_5 , 106–108). There are five loops (loop₁, residues 19–25; loop₂, 35–40; loop₃, 56–69; loop₄, 77–84; and loop₅, 100–105). The major hydrophobic core (core₁) is formed by the docking of helix₁ on the β -sheet. Core₂ is formed by residues from loop₁, loop₂, α -helix₂, α -helix₃ and the first β -strand. This core has no central residues and contains several water molecules. Core₃ is formed by the packing of Leu-63 in loop₃ against residues in the β -sheet (on the opposite face to its packing with helix₁), loop₃ and loop₅. Leu-63 and Leu-89 face each other and form the central residues of core₃.

4.1. Structural integrity on mutation

The crystal structures of several mutants have been obtained at high resolution. Tyr→Phe-78, which removes the -OH that forms buried hydrogen bonds with

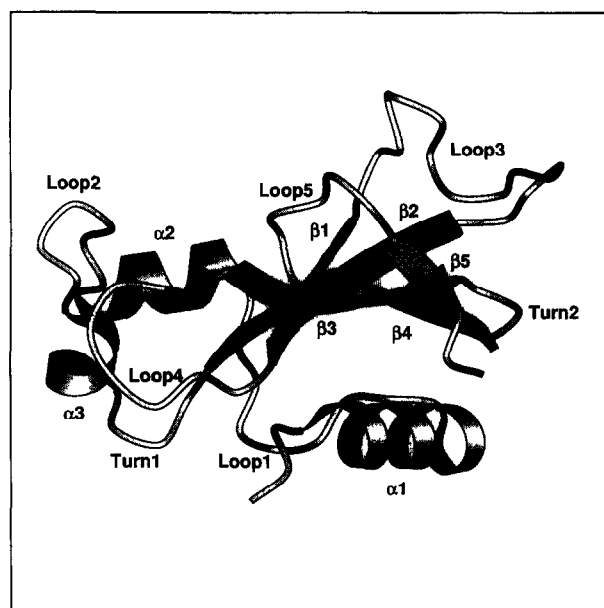


Fig. 3. Structure of barnase.

the backbone CO and NH of Gly-81, is a perfect non-disruptive deletion, with no observable change in structure aside from the removal of the group, and the cavity does not become occupied with water. The same is true for Ser→Ala-91 (Wai Chen and Kim Henrick, unpublished). Mutation of several isoleucine residues to valine in the hydrophobic core gives derivatives that have empty cavities (Ashley Buckle and Kim Henrick, unpublished). In some cases there are no changes of structure, whereas in others there are small rearrangements of neighbouring side chains to give a partial collapse of the cavity. (Similar results on cavity formation have been reported for T4 lysozyme [40].) The integrity of the structure of barnase is thus quite robust in response to small mutations, as is that of T4 lysozyme [41].

5. THE MAJOR TRANSITION STATE FOR UNFOLDING AND THE LATE INTERMEDIATE

5.1. Kinetics and equilibria in folding

Much of the analysis involves urea-mediated unfolding. This fortunately follows simple laws for kinetics and equilibria. The free energy of unfolding, ΔG_U , of many proteins in urea solutions is linearly proportional to [urea]:

$$\Delta G_U = \Delta G_U^{H_2O} - m[\text{urea}] \quad (1)$$

where $\Delta G_U^{H_2O}$ is the free energy of unfolding in water and m is a constant that is proportional to the increase in degree of exposure of the protein on denaturation [42]. The change in free energy of unfolding on mutation is readily measured with precision by urea denaturation by applying eqn. 1 to mutants. Consistent data for barnase are found from experiments using guanidinium

chloride- or thermal-denaturation. Measurements on a large number of mutants has led to a library of interaction energies [23]. Rate constants for unfolding and folding also change with [urea]. For example, the rate constant for unfolding, k_u , is often found [42] to increase with increasing [urea] according to eqn. 2, where $k_u^{\text{H}_2\text{O}}$ is the rate constant in H_2O and mk_u is proportional to the increase in exposure of the protein on going from the folded to the transition state.

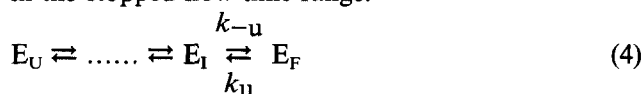
$$\log k_u = \log k_u^{\text{H}_2\text{O}} + m_{ku}[\text{urea}] \quad (2)$$

This simple relationship allows the extrapolation of data to the absence of urea. Andreas Matouschek has now shown from exquisitely precise data (unpublished) that, whereas eqn. 2 describes the kinetics of unfolding of barnase to a high degree of accuracy over short ranges of [urea], deviations from linearity do occur over wide ranges and the data fit a quadratic equation:

$$\log k_u = \log k_u^{\text{H}_2\text{O}} + m_{ku}[\text{urea}] - \beta[\text{urea}]^2 \quad (3)$$

(The changes in $\log k_u^{\text{H}_2\text{O}}$ that are used for the ϕ -value analysis are not significantly affected when using the more complex extrapolation of eqn. 3 instead of the linear extrapolation.)

Complexities in the kinetics of refolding show that there is one major intermediate on the pathway (eqn. 4) in the stopped-flow time range.



There is at least one further intermediate that is lost in the dead time of the apparatus (< 5 ms). Other intermediates certainly occur between E_I and E_F but this is after the rate-determining step for folding. k_u , the observed rate constant for unfolding (in the example of barnase), is the rate constant for the formation of E_I from E_F , which is the rate-determining step for unfolding. k_{-u} is the rate constant for the formation of E_F from E_I . Determination of k_u , k_{-u} and ΔG_U allows the construction of the partial free energy diagram (Fig. 2), which allows the structures of the late folding intermediate, E_I , and the major transition state between E_I and E_F to be probed.

The free energy profile and the transition states for protein folding are more complex than those for a simple chemical reaction in which changes are usually localised to just a few bonds. The transition state in protein folding encompasses much of the molecule, and there are probably conformations of similar energies that will be significantly occupied. Similarly, the free energy profile will contain many minor maxima and minima that are superimposed upon the major features because of small conformational events of low activation energy that occur during the pathway [20].

To date, we have analysed over 120 mutants that involve more than half the residues of the protein, illustrated in Figs. 4–7. Many of the ϕ -values are either close

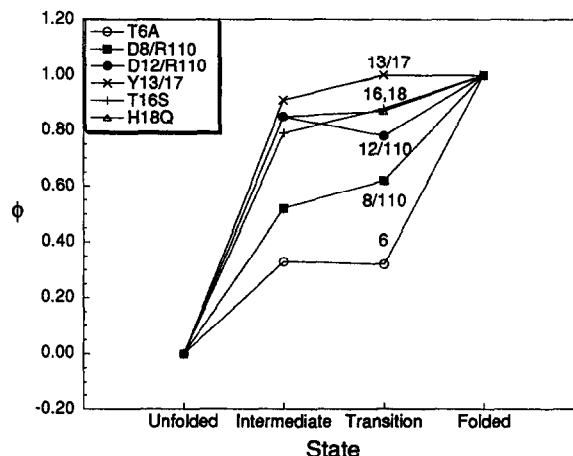
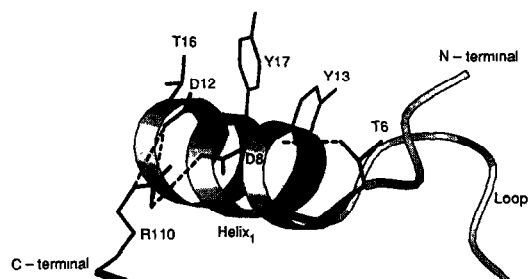


Fig. 4. Residues in α -helix₁ (top) and the ϕ -values found for mutation (bottom).

to 0 or close to 1 for the transition state and intermediate, suggesting that the relevant regions are completely unfolded or completely folded, respectively. Different probes for many regions of the protein give consistent results, as do multiple probes at the same site. There are beautiful gradations of ϕ -values observed as one scans along the structure and the free energy profiles. The overall consistency of ϕ -values indicates that the mutations have not produced changes in the protein that significantly alter the transition state for unfolding or the folding intermediate.

It should be noted that the ϕ -values may be derived both for the absence of urea, since refolding can be performed directly in the absence of urea and the unfolding data are extrapolated to the absence of urea by Eqn. 3, and also in the presence of urea. Values of ϕ are found to be somewhat insensitive to the concentration of urea, and so the general features of the unfolding pathway are not obscured by the presence of urea. This is reasonable on chemical grounds since proteins are unfolded by urea not because the folded state is destabilised but because the unfolded state is stabilised. Urea acts as a denaturant by displacing the equilibrium to favour the unfolded state. We can now describe the sequence of structure formation in the various substructures of barnase.

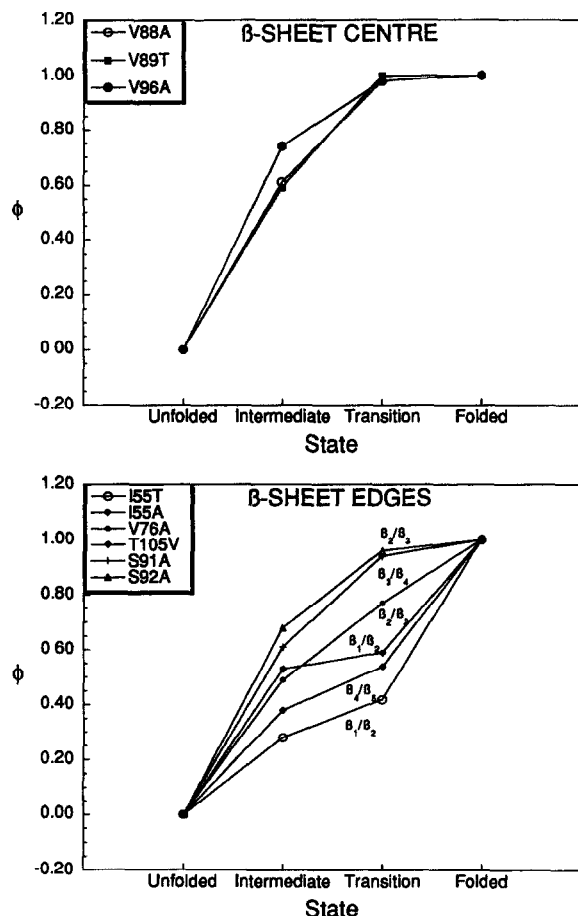


Fig. 5. ϕ -values found for mutation of residues in the β -sheet; centre (top) and edges (bottom).

5.2. α -helix₁ (Fig. 4)

Mutation of the C-terminal region of the helix at residues 16 and 18 gives ϕ -values of close to 1 in the intermediate and the transition state. The same is found for interactions between Tyr-13 and Tyr-17, and Tyr-17 and Thr-16 from double-mutant cycles. This shows that the C-terminus of the helix is present from residues 13–18 in the intermediate and the transition state. The low values of ϕ for mutation of Thr-6 at the N-terminus shows that this is predominantly disordered prior to the final folded state. The gradation from order to disorder from residues 13–8 is nicely seen from a triple-mutant cycle analysis of the interactions of Asp-12 and Asp-8 with Arg-110. The Arg docks onto the helix via a salt bridge that relies on Asp-12 and Asp-8 being in their helical geometry.

5.3. β -sheet (Fig. 5)

Nice gradations of ϕ -values are seen both as the reaction proceeds and on-going from the centre of the sheets to the edges. Three probes obtained from mutations in the centre of the sheet (Fig. 5, top; V88A, V99T, V96A) show consistent progressive formation of structure as

folding occurs, with the centre of the β -sheet being 100% formed in the major transition state (Fig. 5, bottom). The central strand of the five-stranded sheet is β_3 . Its interactions with β_2 and β_4 at the edges have ϕ -values of ~ 0.6 in the intermediate and 1.0 in the transition state. The ϕ -values become lower further away from the central strands (β_1/β_2 , β_4/β_5).

5.4. Core₁ (Fig. 6)

The exquisite increases in ϕ -values are again seen as the reaction progresses and as the probes become closer to the centre of the major hydrophobic core. The terminal methylene group of I88 at the centre of the core has strong interactions in the intermediate which approach a ϕ -value of 1 in the transition state, whereas that of I76 at the edge has ϕ -values of close to zero for both states.

5.5. Loops 1–4 (Fig. 7)

These fall into two clear-cut classes. Loops 1, 2 and 4 have ϕ -values of 0 throughout the reaction, showing that they do not fold until after the rate-determining transition state. Loop₃ folds early.

Many other parts of the structure have been investigated [25], and the results so far may be summarised. The β -sheet, the last two turns of α -helix₁, the guanosine binding loop (part of loop₃) and the docking of the

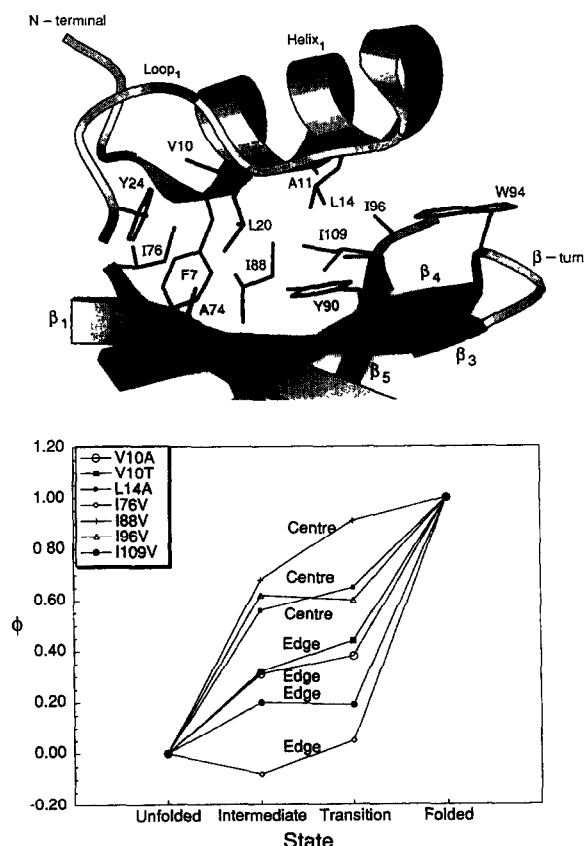


Fig. 6. Residues in the major core (top) and the ϕ -values found for mutation (bottom).

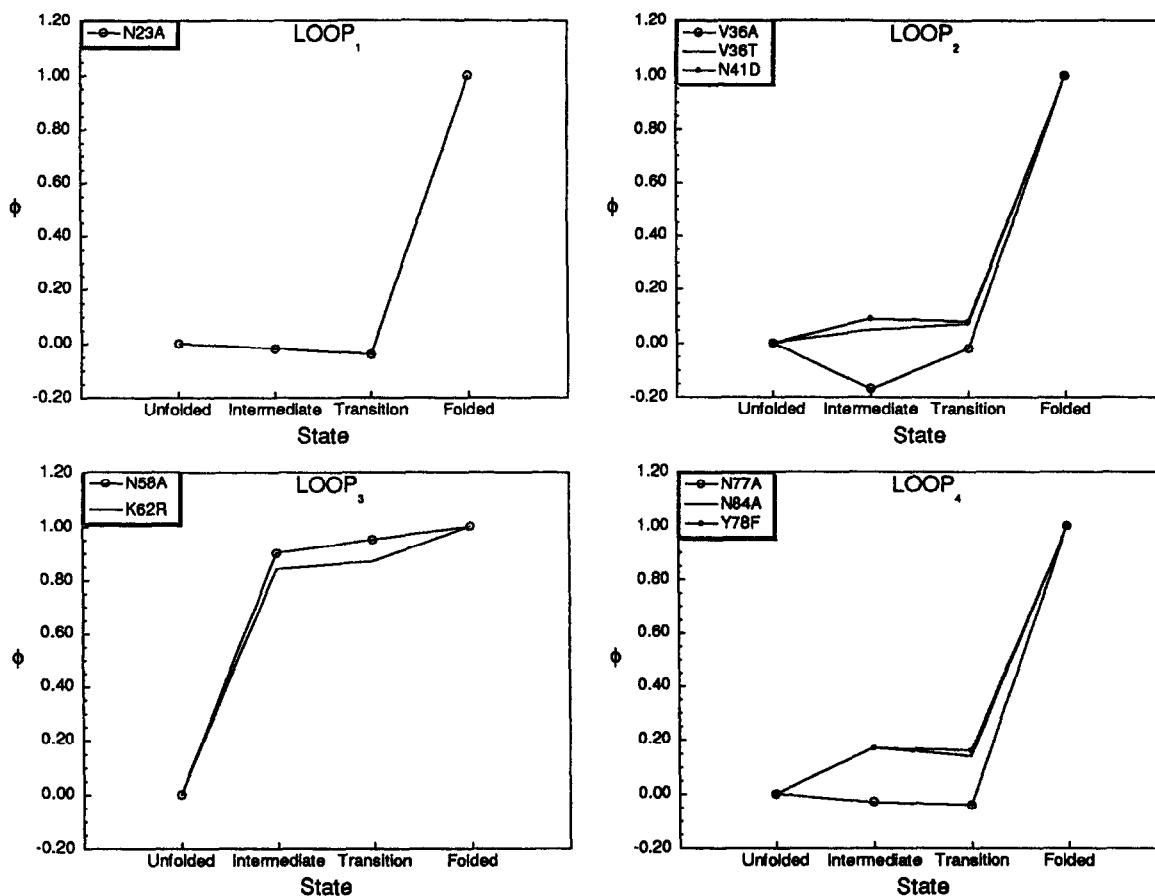


Fig. 7. The ϕ -values found for mutation of the loops in Fig. 3.

C-terminal region of the protein, are formed early in the folding reaction. The N-terminal turn of α -helix₁ and the β -sheet are consolidated throughout the folding reaction. The hydrophobic packing of α -helix₁ against the β -sheet, which forms hydrophobic core₁, and the packing of the guanosine binding loop against the β -sheet, which forms hydrophobic core₃, are present early, but are consolidated throughout the folding reaction. This consolidation affects mainly hydrophobic core₁, especially the interactions between α -helix₁ and the β -sheet, and this consolidation seems to be part of the rate-limiting step in the reaction. The first turn of α -helix₂, part of loop₁ and loop₄, and hydrophobic core₂, are not formed until the protein is fully folded. The second turns of α -helix₂ and α -helix₃, are formed early in the folding pathway but the packing against each other and with the rest of the protein does not occur until the protein is fully folded.

5.6. Information on the intermediate from H/D exchange

Backbone NH groups that are involved in hydrogen bonds in either secondary structure, such as helices or sheets, or in some tertiary interactions, may be protected against H/D exchange with solvent H₂O or D₂O [43]. This can be used as a probe to monitor the appear-

ance of secondary structure during folding using rapid quenching and sampling techniques [44] and two-dimensional ¹H NMR spectroscopy to resolve events at individual positions [11–13,45,46]. Application of this method to barnase [26,38] confirms the deductions from ϕ -values that there is a folding intermediate with the following characteristics: (i) helix₁ is complete from residues 10–18; (ii) the interactions between all β -strands are also present; (iii) part of loop₂ is not formed; (iv) part of loop₃ is formed; and (v) some specific tertiary interactions are not made. There is also the important additional information that there is: (i) early formation of the C-terminus of helix₂; (ii) early formation of helix₃; (iii) early formation of several β -turns (46–49, 101–104 in loop₅); and (iv) partial formation of loop₅.

Exchange and protein engineering give self-consistent results.

5.7. Rationally engineering the accumulation of the folding intermediate for direct study

The major folding intermediate has been engineered by Jesús Sanz (unpublished) to accumulate using the following logic. According to Eqn. 1, the free energies of the different states of the protein in urea should each decrease linearly with [urea] by the law:

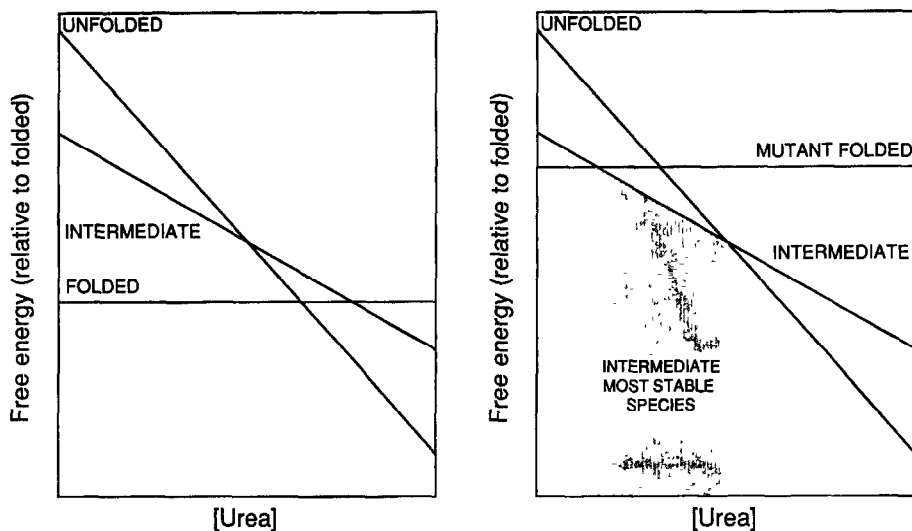


Fig. 8. Strategy for accumulating the intermediate.

$$\Delta G_i = \Delta G_i^{\text{H}_2\text{O}} - m_i[\text{urea}]$$

The value of m_i is proportional to the solvent exposure of the state, and so $m_U > m_I > m_F$ for the unfolded, intermediate, and folded states, respectively, since they represent progressive steps of unfolding. The relative energies of those states thus follow the pattern of Fig. 8. For wild-type enzyme, the energy of I is always higher than that of U or F (Fig. 8, left), but, if we can engineer a mutation that lowers the stability of the folded state without lowering the stability of the unfolded and intermediate, then, according to the right-hand side of Fig. 8, the intermediate should accumulate in a concentration range of urea close to that of the unfolding transition. We have, of course, identified such mutations from the ϕ -value analysis. Substituting a number of these into barnase causes the intermediate to accumulate at moderate concentrations of urea. For example (Fig. 9), CD and fluorescence titration shows that the tertiary structure of the mutant Ile-4→Ala/Ile-51→Val melts at 2.9 M urea, while the remaining helical secondary structure melts at 3.6 M. Further, differential scanning calorimetry (J.S. Johnson and C. Johnson) shows that the mutant melts at low pH via a well-resolved three-state process in which the intermediate accumulates.

We are now ready to study the structure of the intermediate directly by NMR and other spectroscopic methods.

5.8. Are there parallel pathways of folding?

The ϕ -values also provide indirect information on two of the classical problems on characterising intermediates: is the intermediate on the reaction pathway and are there parallel pathways?

Intermediates can occur that are not on the pathway but are dead-end side products that have to revert to starting materials to form the final product. These inter-

mediates must have non-covalent bonds in them that are not present in the final product and that have to be broken. Some of these bonds would thus have values of ϕ in the intermediate that are higher than in the following transition state or even greater than 1, however, in all cases there is a uniform progression of ϕ as the reaction proceeds. This is the behaviour expected of an intermediate that is on the pathway.

There could be parallel folding pathways that generate a mixture of intermediates. If so, then the ϕ -values would be weighted means of those for the different intermediates. A heterogeneous collection of structures would generate many fractional values of ϕ since some of the intermediates would have parts of structure formed and others would have those parts unformed. The majority of intermediates have values of ϕ close to 0 or 1.0, especially in the transition state. Further, values of ϕ from a triple mutant study involving residues from helix₁ interacting with the N-terminus of barnase (Asp-8/Asp-12/Arg-110) show that there is high cooperativity in the intermediate that is indicative of an intermediate on a sequential rather than parallel pathway [30].

The regions that do have fractional regions of ϕ are in a state of flux. This could result from those regions having a mixture of folded and unfolded populations, or the interactions being in the process of being formed as part of the rate-determining step. Many of the fractional ϕ -values are in hydrophobic core₁, and this suggests its consolidation, via docking of α -helix₁ and the β -sheet, is part of the rate-determining process.

The ϕ -value analysis is currently being extended in several directions. The first is by the analysis of earlier intermediates (see section 6). A second is by the analysis of a deeper layer of mutations. Jacqui Matthews is analysing the further substitution of Gly for Ala at various positions that have already been mutated to Ala.

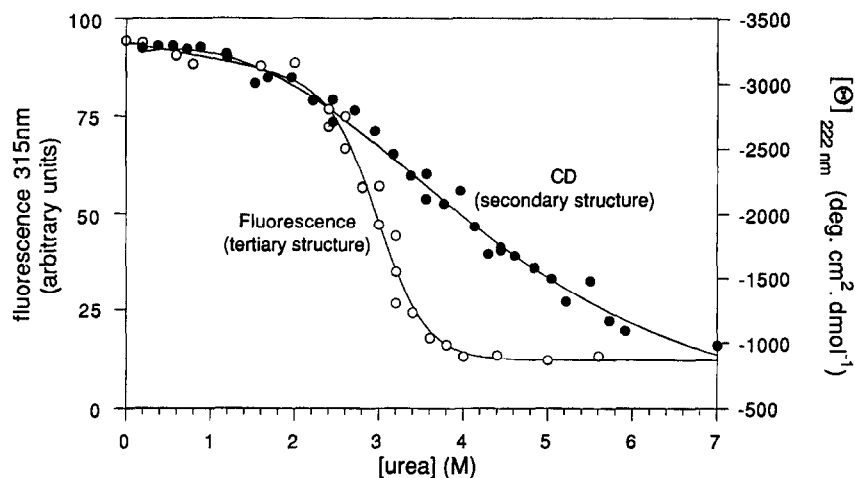


Fig. 9. Differential unfolding of tertiary and secondary structure for the mutant I4A/I51V in which Ile-4 has been mutated to Ala and Ile-51 to Val. The fluorescence at 315 nm monitors the tertiary structure and the ellipticity at 222 nm mainly the helical content of the secondary structure.

This is giving more detail about the order of events in formation of structure as the reaction pathway becomes more distorted from that of wild-type barnase. Third, Andreas Matouschek is analysing the changes in m_{ku} on mutation, which is giving subtle details on the order of events.

6. DETECTION OF EARLIER INTERMEDIATES

The next challenge is to detect intermediates prior to the major late intermediate described in detail above. These occur on a time scale that is too short for the H/D exchange measurements monitored by quenching and sampling. The protein engineering approach can, however, be used with any rapid reaction technique. To this end, Mikael Oliveberg is applying pressure-jump kinetics, a relaxation method with a time resolution in the 10–100 μ s region, to refolding. Martin Long is applying temperature-jump, with a time resolution down to a μ s, to unfolding.

7. STRUCTURE IN THE UNFOLDED STATE

7.1. Local and early folding events

It is clear that two of the questions ‘What is the order of events during folding?’ and ‘Are there parallel pathways of folding?’ can be answered by the above types of experiments. These can be extended to shorter times scales for the ϕ -value analysis by employing rapid reaction techniques. ‘Are there stop and start signals for elements of structure?’ is being answered by statistical survey [47,48] and measurement of energetics at equilibrium [28]. In particular, the beginning and end of a helix seem well-defined by the choice of cap residues. The three questions: ‘Do segments of structure form independently?’, ‘What drives structure formation?’ and ‘Are there initiation sites?’ need additional methodology, described next. This is because the final structure

of a protein is stabilised by both short- and long-range interactions. One approach is to examine fragments of a protein [49] in isolation to understand what drives local structure formation, and the stabilising effect of tertiary interactions on those segments of secondary structure [50].

7.2. Detecting low levels of residual structure in peptide fragments: α -helices

It is important to know whether isolated fragments of proteins have a propensity to take up the secondary structure they occupy in the folded state. If they do, then this shows that local interactions are important in the folding pathway, and that local structure formation can act as foci for folding. It is now a routine matter to detect secondary structure in peptide fragments using NMR or CD when that structure accumulates. It is a challenge, however, to detect low levels of structure. The addition of trifluoroethanol (TFE) often induces peptides that have a helical propensity to take up this conformation, but sometimes forces peptides that should be in a β conformation to become helical! Javier Sancho [51] has introduced a procedure for measuring low amounts of helical structure of peptides in water and quantifying the helical tendency. The fraction of helical content on the increasing addition of trifluoroethanol is measured from the CD signal at 222 nm and is fitted to the equivalent of eqn. 1. That is:

$$\Delta G_{helix} = \Delta G_{helix}^{H_2O} - m [TFE]$$

assuming that the free energy of formation of the helix, ΔG_{helix} , is linearly proportional to [TFE]. The peptide fragment 1–36 from barnase that contains the two major helices appears from its CD spectrum to be largely disordered. Titration with TFE was found to induce helix formation and also to detect some helical content in water [51]. The helical region was shown by NMR to reside mainly in the region of residues 10–18

(the major helix spans 6–18 in the native protein but primarily 10–18 in the folding intermediate). Importantly also, the helix in the fragment in TFE does not extend beyond the region found in the native protein, showing that the stop and start signals (the ‘caps’) are effective. There is no theoretical basis for the TFE titration method at present, but Alan Jasonoff is working on the theoretical and experimental basis of the method since it does appear to be potentially very useful. The method does appear to work in practice. For example, Alistair Kippen has shown from a CD/TFE titration that the peptide 1–22 of barnase has about 1% helical structure in water and that it becomes largely helical at high [TFE]. Important evidence that the TFE procedure is not artefactual comes from measuring the equilibrium constant for helix formation as a function of pH. Residue His-18 is the C-cap of the helix and has been found to have its pK_a raised from 6.6 in the unfolded state in the intact protein to 7.8 in the folded state by the influence of the helix dipole and hydrogen bond with the CO of Gln-15 [52–54]. The TFE measurements give a pK_a of 6.6 in water and 7.5 in TFE solution for His-18 in the fragment.

7.3. Detecting low levels of residual structure in larger fragments?

The fragment 23–110 appears from its CD spectrum to be largely disordered. This fragment has all the residues necessary for binding to barstar, the polypeptide inhibitor of barnase, and there is preliminary evidence that the fragment does bind to barstar (Alistair Kippen, unpublished). The small (1–22) and large (23–110) fragments also bind to each other very rapidly (with a second-order rate constant of $\sim 10^5 \text{ s}^{-1} \cdot \text{M}^{-1}$) and tightly to give a catalytically active complex. This suggests that a small fraction of the larger fragment does exist in the native conformation or has a ready propensity to do so.

7.4. Structure in the unfolded state

It is of complementary interest to determine what residual structure exists in the nominally unfolded state of a protein. Recently Neri et al. [55] have shown that it is possible to measure residual structure in an unfolded state by using magnetization transfer NMR. Dario Neri and the Wüthrich laboratory have provided invaluable assistance to us (Stephane Vuilleumier, Vickery Arcus, Andrea Hownslow and Mark Bycroft) in assigning the denatured state of barnase in acid and a suitable mutant in urea solution. About 30% of the ^1H signals in the denatured state have been assigned so far, and so there is every prospect of solving these structures, as well as that of the accumulated late folding intermediate, by NMR in the near future.

7.5. Conclusions from the studies on fragments

Barnase appears to be able to fold independently in separate parts that can associate. There are three impor-

tant conclusions from these result. First, as pointed out by Wetlaufer [2] and found earlier for a helical peptide of RNase A [7], the independent formation of small segments of structure cuts through the Levinthal paradox. Second, it shows that secondary structure formation in barnase can precede the docking of the major α -helix onto the β -sheet. Third, Moulton and Unger [56] have developed a model for protein folding in which formation of local secondary structure is driven by the burial of hydrophobic surface area. John Moulton predicted independently (personal communication) that residues 10–21 in barnase constitute an initiation site for folding because they bury a high degree of hydrophobic surface area on forming an α -helix and, similarly, the β -hairpin formed by strands 3 and 4 (residues 85–99) buries considerable hydrophobic surface area.

8. FURTHER KINETIC TECHNIQUES

8.1. Comparative merits of the major procedures

The protein engineering method (kinetic and equilibrium measurements on mutants) is, in many ways, the most general and informative. The method can be applied to almost every side chain in a structure. Transition states can be studied as well as intermediates. Energetics are measured and hence the extent of structure formation may be determined. A variety of spectroscopic and rapid-reaction techniques can be applied that give a continuous record of data on time scales that are shorter than those for rapid quenching and sampling. Trapping of transient intermediates by the NMR-monitored H/D-exchange method is a splendid procedure for identifying individual interactions on a time scale of milliseconds or greater. It is, however, limited only to NH groups, and these are restricted to only those positions that are sufficiently well-protected against exchange. The progress curves are often multiphasic and it is difficult to accumulate sufficient data points to deconvolute the complex kinetics. Equilibrium H/D exchange is a powerful technique, and has been applied in conjunction with other spectroscopic techniques to lysozyme [57,58].

8.2. A new twist to disulphide bridges

The trapping of the different disulphide-bridged intermediates in the refolding of reduced bovine pancreatic trypsin inhibitor under oxidizing conditions has been of seminal importance in the history of protein folding [8]. Whereas those working on the folding of proteins that have natural disulphide bridges are now busily removing them to facilitate kinetic studies, we are perversely introducing them into barnase, but for a different purpose. Jane Clarke [59] has shown that linking together parts of the protein that are predicted to unfold before the major unfolding transition state dramatically slows down the rate of unfolding. Conversely, linking regions that remain together in the transition state has

a much smaller effect on the kinetics of unfolding. Not only do these data support the predictions on the structure of the transition state but they also suggest which parts should be stabilized in proteins that are used in biotechnological processes where the kinetics of denaturation are important.

9. CONCLUSIONS

The studies on barnase have given for the first time a detailed description at the level of individual side chains of the structures of a folding intermediate and the major transition state for unfolding/refolding. What generalisations can we make from these novel data, aside from the measurements of individual interaction energies that are discussed elsewhere [28]? The broad conclusions relate to correlations between the burial of hydrophobic surface area, initiation events, and the formation of secondary and tertiary structure [27]. The C-terminal part of the major α -helix forms early in folding of the intact protein, and also partially folds in a fragment, driven by hydrophobic interactions. This could be a putative initiation site for folding. The central β -hairpin is also a very strong candidate for an initiation site because it buries considerable hydrophobic surface area on its formation. It is observed that all the regions that fold early interact extensively with the β -sheet. These interactions are primarily hydrophobic and involve the burial of very extensive hydrophobic areas in which multiple, close, hydrophobic-hydrophobic contacts are established around a central group of aliphatic residues. The major principle inferred from the results is that there appears to be a correlation between burying hydrophobic surface area and early folding events. The results are consistent with one of the earlier events in protein folding being the local formation of native-like secondary structure elements driven by local hydrophobic surface burial. Folding is then driven by the further burial of hydrophobic surface as the major elements of secondary structure, helix₁ and the β -sheet, dock and consolidate the hydrophobic core in a step that is partly rate determining.

It should be evident from the experiments described in this lecture that extension of direct NMR methods and analysis of mutant proteins by further kinetic and biophysical techniques should fill in many of the gaps in Fig. 1, and so obtain a detailed picture of the folding pathway and evidence on the principles by which it is directed.

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