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Pathway and Stability of Protein Folding

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Pathway and stability of protein folding

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SUMMARY

We describe an experimental approach to the problem of protein folding and stability which measures interaction energies and maps structures of intermediates and transition states during the folding pathway. The strategy is based on two steps. First, protein engineering is used to remove interactions that stabilize defined positions in barnase, the RNAse from *Bacillus amyloliquefaciens*. The consequent changes in stability are measured from the changes in free energy of unfolding of the protien. Second, each mutation is used as a probe of the structure around the wild-type side chain during the folding process. Kinetic measurements are made on the folding and unfolding of wild-type and mutant proteins. The kinetic and thermodynamic data are combined and analysed to show the role of individual side chains in the stabilization of the folded, transition and intermediate states of the protein. The protein engineering experiments are corroborated by nuclear magnetic resonance studies of hydrogen exchange during the folding process. Folding is a multiphasic process in which α-helices and β-sheet are formed relatively early. Formation of the hydrophobic core by docking helix and sheet is (partly) rate determining. The final steps involve the forming of loops and the capping of the N-termini of helices.

1. INTRODUCTION

Molecular recognition in biology is principally controlled by non-covalent bonds: van der Waal's. electrostatic, hydrogen bonding and the hydrophobic effect. These interactions are responsible for information transfer from DNA to RNA to proteins, the assembly of macromolecules, the binding of ligands and the three-dimensional structure of proteins. The essence of enzymic catalysis is the use of binding energy and complementarity of enzyme–substrate interactions (Fersht 1985). It is now possible to study non-covalent interactions within proteins directly by using protein engineering (Winter *et al.* 1982). We are now applying protein engineering methods to the problem of protein folding: the prediction of the three-dimensional tertiary structure of a protein from its linear sequence of amino acids. Protein folding, like any chemical process, has two components, kinetic and thermodynamic: the pathway of folding and the stability of the folded state. It is not yet possible to solve either of these components by computational methods *de novo*. As far as kinetics is concerned, too many conformations occur between the denatured protein and the folded structure to be searched at random. It is not even possible today to calculate whether a known folded structure is stable with respect to its denatured state. Experimental data are necessary for both the thermodynamic and kinetic processes.

We have developed a strategy using protein engineering to provide necessary experimental data on both pathway and stability (Matouschek *et al.* 1989, 1990). First, we use site-directed mutagenesis to remove interactions that stabilize parts of proteins and then measure the changes in stability. This provides empirical thermodynamic data that can be used in redesigning enzymes and provides a data base for testing theoretical procedures. Second, we perform kinetic measurements on folding and unfolding of the mutated enzymes to measure the changes in activation energies and equilibrium energy levels. The relationship between changes in activation and equilibrium energies may be used to map the structures of transition states and intermediates (Matouschek *et al.* 1989, 1990; Matouschek & Fersht 1991).

Our protein of choice as a paradigm for protein folding studies is barnase, an extracellular ribonuclease from *Bacillus amyloliquefaciens*. It is a small monomeric enzyme of 110 residues, with a relative molecular mass of M_r of 12382. It is composed of a single domain of about the size expected for a folding unit of a large protein, and has α and β secondary structure. Barnase undergoes reversible solvent- and thermally induced denaturation, closely approximating to a two-state transition. The crystal structure of the protein has been solved at high resolution (Mauguen *et al.* 1982) and its solution structure has also been elucidated by high-field proton nuclear magnetic resonance (NMR) spectroscopy (M. Bycroft, unpublished data). Importantly for kinetic experiments, there are minimal effects from the rates of proline isomerization. The kinetics of folding of proteins is often complicated by the slow and frequently rate-determining isomerization of proline residues (Schmid *et al.* 1986). It is expected that some

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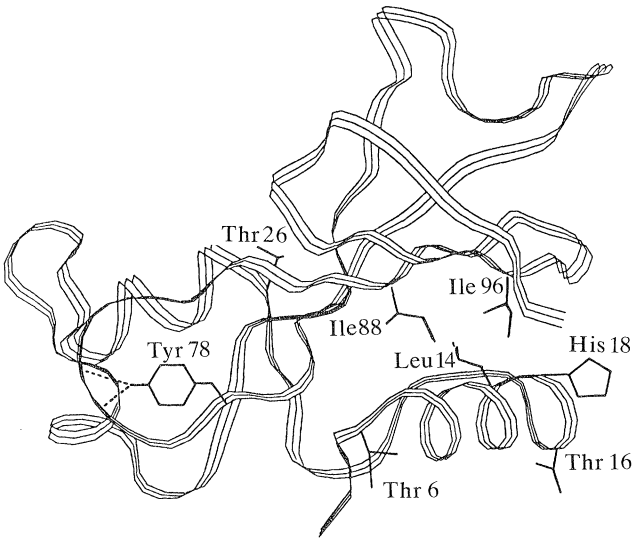

Figure 1. Sketch of barnase, showing the initial residues mutated in this study.

5–20% of each of the prolines in denatured protein should exist in the *cis* conformation and that the conformations should interconvert with a half-life of tens of seconds (Brandts *et al.* 1975). Barnase has just three proline residues and they are all *trans* in the native structure. Consequently, the major phase in the refolding of barnase is the fraction ($\approx 75\%$) of unfolded protein that has all its prolines in the *trans* conformation in solution. The gene for this enzyme has been cloned and can be expressed in *Escherichia coli* (Paddon & Hartley 1987). The tertiary structure of barnase comprises a C-terminal five-stranded anti-parallel β-sheet (residues 50–55, 70–75, 85–91, 94–101 and 106–108) with two N-terminal α-helices, the major one (residues 6–18) packed against its face and the other (26–34) against its edge. The hydrophobic core of barnase is formed from the non-polar side chains of the major α-helix interdigitating with those of the β-sheet (figure 1).

2. EXPERIMENTAL APPROACH

Protein denaturation is monitored by the large decrease in the fluorescence of the tryptophans. Our main method of inducing denaturation is by the addition of urea. The data are extrapolated to 0 M [urea] by standard procedures (Pace 1986). We find the results thus obtained are independent of whether denaturation is urea-, guanidinium- or temperature-induced (Kellis *et al.* 1989). Kinetics of denaturation and renaturation are obtained by stopped-flow fluorescence and [urea]-jumps (Matouschek *et al.* 1989, 1990).

3. FOLDING INTERMEDIATE

An intermediate, or series of intermediates, was detected in the refolding of barnase (Matouschek *et al.* 1990). The intermediate does not appreciably accumulate at medium (4–5 M) to high concentrations of urea, but does do so at low concentrations (figure 2). There is growing evidence that the refolding of proteins *in vitro* may proceed, in general, *via* transient intermediates (Udgaonkar & Baldwin 1988; Roder *et al.* 1988). Earlier work has shown the existence of a state, termed the molten globule, which is stable under mildly denaturing conditions (acid pH, moderate concentrations of denaturants, or high temperatures (Ptitsyn *et al.* 1990)). An outstanding question is what is the structure of these intermediates, and do they have the common features for the molten globule. We can analyse the structure of the intermediate and also the transition state for its formation from the folded state (the 'unfolding' transition state) by protein engineering methods (Matouschek *et al.* 1989, 1990; Matouschek & Fersht 1991).

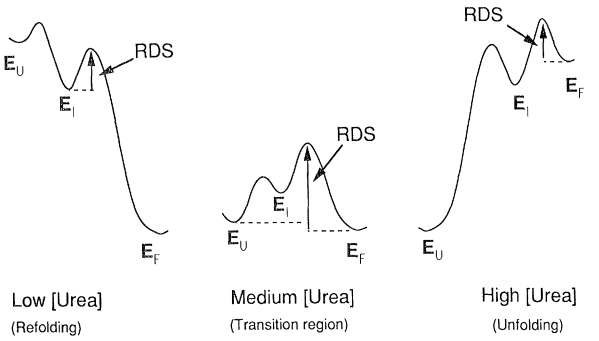

Figure 2. Minimal free energy profiles that describe the unfolding and refolding of barnase at low (< 2 M), medium (4–5 M) and high (> 6 M) [urea].

4. DIFFERENCE ENERGY DIAGRAMS

It was shown how to analyse the role of binding energy of groups at the active site of the enzymes by determining the free energy profiles of wild type and mutants throughout the reaction, and measuring the differences between the two (Wells & Fersht 1986; Fersht *et al.* 1987). The 'difference energy' diagrams readily give a qualitative, and even quantitative (Fersht 1988), picture of the contribution of each side chain to binding and, hence catalysis, throughout the reaction. An identical approach is now used to analyse protein folding. First, suitable mutations must be chosen. Ideally, the target side chain is involved in just one interaction with one other side chain, preferably a weak interaction. The moiety that makes that interaction is then deleted by mutagenesis. Suitable changes are Ile → Val, Thr → Ser and Tyr → Phe. It is important not to remove a residue that solvates a buried charge as the charged residue in the mutant may cause serious structural changes in becoming solvated. If these mutations are non-disruptive, then the changes in energy on mutation reflect the interaction of the target side chain. Next, the free energy profiles of the folding pathway wild-type must be constructed. The kinetics of folding and unfolding fit that expected for a simple two-step reaction but this is just part of a more general scheme which can have multiple intermediates. The intermediate we see is the slowest formed, or a mixture of such intermediates.

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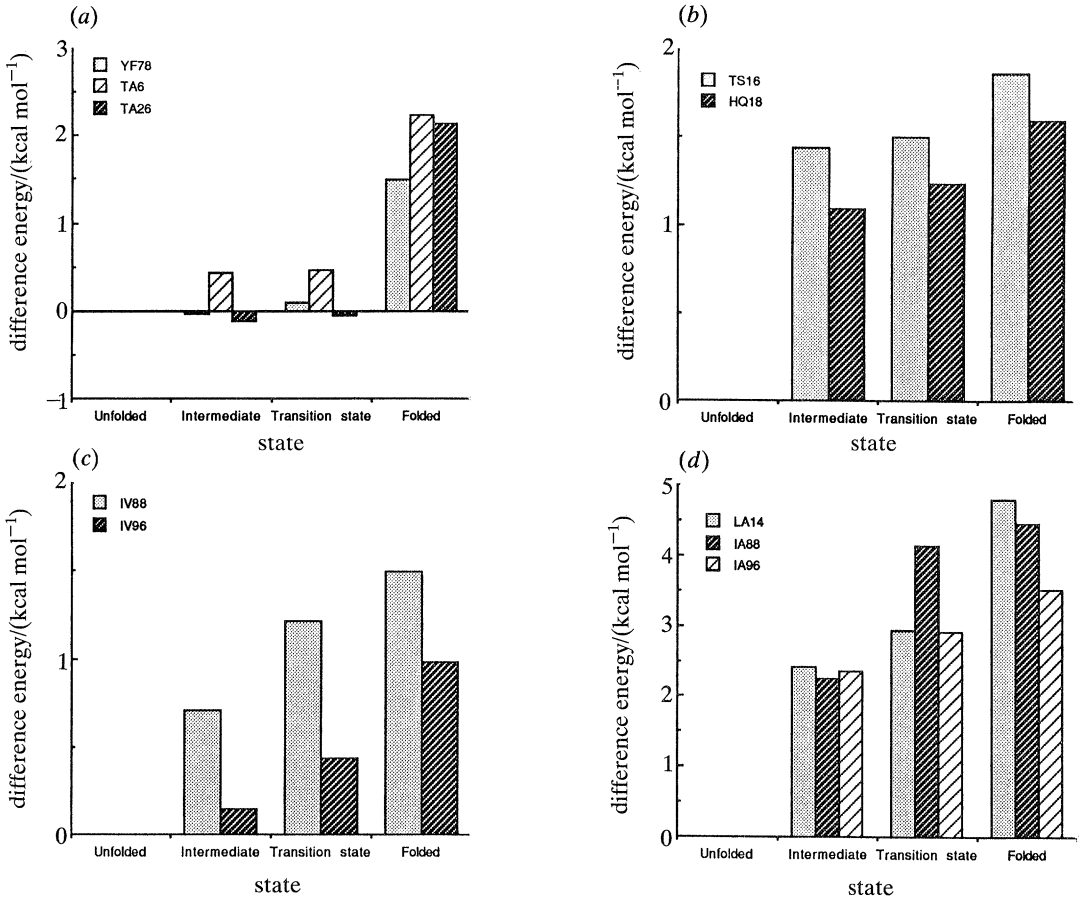

Figure 3. Difference energy diagrams for the folding of the mutants: (*a*) Tyr → Phe 78, Thr → Ala 6 and Thr → Ala 26; (*b*) Thr → Ser 16 and His → Gln 18; (*c*) Ile → Val 88 and Ile → Val 96; Leu → Ala 14, Ile → Ala 88 and Ile → Ala 96.

5. INITIAL DESCRIPTION OF INTERMEDIATE AND UNFOLDING TRANSITION STATE FROM PROTEIN ENGINEERING METHODS

Difference energy diagrams for a variety of mutants, each chosen to probe specific points of the structure, are plotted in figure 3. We sometimes describe the changes in difference energies in terms of a function ϕ. For example, if the change in free energy of unfolding on mutagenesis is $\Delta\Delta G_U$, and the change in activation energy on mutation for the rate constant of unfolding is $\Delta\Delta G_U^{\ddagger}$, then $\phi_U = \Delta\Delta G_U^{\ddagger}/\Delta\Delta G_U$. Quantitative interpretation is complicated because the unfolded state is the reference structure for each mutant. The difference energies are not, therefore, the true differences in non-covalent energy between the wild-type and mutant enzymes because the measured energies for each mutant differ from the true by a term which is the difference in free energy of the unfolded wild-type protein and the specific mutant (Matouschek *et al.* 1990; Matouschek & Fersht 1991). This does not, however, affect the interpretation of two extreme, but very common, cases: (i) the target side chain is as exposed in the transition state or intermediate as it is in the unfolded protein, the difference energy = 0 = ϕ; (ii) the target side chain makes the same interaction in the transition state or intermediate as it does in the folded protein, the difference energy is thus the same as in the folded state, and $\phi = 1$. The interpretation of fractional values of ϕ is affected by the differences in energy of the unfolded states but we have shown where this may be ignored or where the changes can be analysed more quantitatively (Matouschek *et al.* 1989, 1990; Matouschek & Fersht 1991). The roles of the different side chains in stabilizing the different states are seen immediately on examining the difference energy diagrams (figure 3).

(*a*) *Formation of the N-termini of the helices and a major loop is a very late step in folding*

Tyr 78 stabilizes a major loop by the —OH group forming hydrogen bonds with the > NH and > C = O of Gly 81 in the folded state. It is seen on mutation of Tyr → Phe 78 that all the stabilization energy of the hydrogen bonds is lost in both the transition state for unfolding and in the intermediate. The —OH of Thr 26 acts as the N-cap of the second helix, forming hydrogen bonds to the > NHs of residues 27–29 (Serrano & Fersht 1989). On mutating Thr → Ala 26, it is seen that all the energy of the N-cap is lost in the transition state for unfolding, and the same is seen to happen in the intermediate. Thr 6 forms the N-cap of the first helix. On mutating Thr → Ala 6, 80% of the difference energy is lost in the transition state for unfolding and the same energy is lost in the intermediate as in the transition state.

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(*b*) *Formation of the C-terminus of the major α-helix is an early event in folding*

The charge on the protonated form of His 18 makes a coulombic interaction with the dipole of the α-helix from residues 6–18 as well as there being a specific hydrogen bond between an imidazole > NH and the > CO of Gln 15. The interaction energy is thus a probe of the integrity of the C-terminus of the helix. Most of this interaction energy is maintained in the transition state and in the intermediate. The γ-methyl of Thr 16 makes a very strong hydrophobic interaction with the aromatic ring of Tyr 17. This is maintained in the transition state for unfolding and in the intermediate. Both probes give consistent results.

(*c*) *Formation of the hydrophobic core is* (*partly*) *rate determining*

Mutation of Ile → Val 96 and Ile → Val 88 probes the hydrophobic core. Some of the energy of the hydrophobic core is lost in the transition state for unfolding, possibly 10–30 % depending on the particular location. Even more energy is lost in the intermediate. The energy changes in the intermediate are midway between those of the unfolded and folded states. In this situation, the changes in difference energies do reflect the true energy changes (Matouschek *et al.* 1989). Consolidation of the hydrophobic core is part of the rate determining process.

6. EXTENSION OF PROTEIN ENGINEERING APPROACH: COSMIC ANALYSIS

There are two limitations in the approach described so far. The first is that it works best for mutation of just simple interactions. Adventitious structural changes may occur when many interactions are broken on mutation and cause a reorganization energy term in the equations. The second is that we assume that the retention of an interaction energy implies retention of the specific interaction. Although this will be true in most cases, it could be that different interactions of similar energy to those in the folded state are taken up in other states. We have now extended the range and reliability of the protein engineering procedure by using the COSMIC (Combination of Sequential Mutant Interaction Cycles) technique in which a series of double-mutant cycles is constructed. In each cycle, the side chains of two amino acid residues that interact in the folded state are mutated separately and together. The technique of double-mutant cycles was introduced to detect interactions between groups at the active site of the tyrosyl-tRNA synthetase (Carter *et al.* 1984; Lowe *et al.* 1985). A formal analysis has been given for their rigorous application to energy changes on protein folding (Serrano *et al.* 1990). Two or more (Horovitz & Fersht 1990), residues, X and Y, that interact are identified from examination of the structure of protein. The two residues 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 for $\text{E-X} \rightarrow \text{E}(\Delta G_{\text{E-X}\rightarrow\text{E}})$ from the free energy change for $\text{E-XY} \rightarrow \text{EY}(\Delta G_{\text{E-XY}\rightarrow\text{EY}})$ causes the interaction energies of X and Y with the rest of the protein to cancel out. An interaction energy, $\Delta G_{\text{int}} = \Delta G_{\text{E-XY}\rightarrow\text{EY}} - \Delta G_{\text{E-X}\rightarrow\text{E}}$, is obtained which is equal to

$$\Delta G_{\text{int}} = G_{\text{X}\cdots\text{Y}} - \Delta G_{\text{X}\cdots\text{w}} - \Delta G_{\text{Y}\cdots\text{w}}, \quad (1)$$

where $G_{\text{X}\cdots\text{Y}}$ is the interaction energy between X and Y, $\Delta G_{\text{X}\cdots\text{w}}$ is the increase in solvation energy of X on removal of Y, and $G_{\text{Y}\cdots\text{w}}$ is the increase in solvation energy of Y on removal of X (Serrano *et al.* 1990). Equation (1) still holds if there is disruption of the structure of the enzyme on mutation of, say, 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 out, reducing their importance to second-order effects.

A quantity, ϕ_{int}, may be defined that is equal to $\Delta\Delta G_{\text{int(X)}}/\Delta\Delta G_{\text{int(F)}}$, where $\Delta\Delta G_{\text{int(F)}}$ is the value of $\Delta\Delta G_{\text{int}}$ in the folded state and $\Delta\Delta G_{\text{int(X)}}$ is that in any other state on the folding pathway. $\phi_{\text{int}} = 1$ means that the inteaction energy is fully maintained in state X whereas $\phi_{\text{int}} = 0$ indicates complete loss. Importantly, fractional values of ϕ_{int} may be simply interpretable. Where the interaction arises from a direct contact, such as a van der Waal's interaction, the two residues have to be within close contact for a significant energetic contribution. Thus appreciable values of $\Delta\Delta G_{\text{int}}$ from the double-mutant cycles show unambiguously that the residues continue to interact in the other state, X. It is possible, however, that the mode of interaction changes.

This approach has been applied to the major helix of barnase (Horovitz *et al.* 1991). The γ-methyl group of Thr 16 interacts with the face of the aromatic ring of Tyr 17 which, in turn, makes an aromatic–aromatic interaction with the side chain of Tyr 13 (Serrano *et al.* 1991). The side chain of Asp 12 is linked to that of Asp 8 via a salt bridge with the guanidinium group of the side chain of Arg 110, the C-terminal residue as shown in figure 2 (Horovitz *et al.* 1990). Tyr 17 makes a large number of interactions within the helix with Tyr 13 and Thr 16. Mutation to Ala 17 is, accordingly, very radical. Tyr 13, as well as its interaction with Tyr 17, makes extensive interactions with the side chain of Pro 21, the main chain atoms of Lys 19, Leu 20 and Pro 21. Mutation of Tyr → Ala 13 is thus a radical change that removes many interactions, including those with residues outside the helix. Mutation of Arg 110 breaks bonds to both Asp 8 and Asp 12. Mutation of Asp 8 affects the energetics of the Asp 12–Arg 110 interaction whereas mutation of Asp 12 affects the energetics of the Asp 8–Arg 110 interaction (Horovitz *et al.* 1990). It is seen in figure 4, however, that the results of the double-mutant cycles are quite clearcut. The value of the energy of the Tyr–Tyr interaction is hardly changed on going from the folded to the transition to the intermediate. This shows that the centre of the helix is already fully formed in the folding intermediate on the folding pathway. The Asp 12–Arg 110 interaction is weakened somewhat during unfolding, and the Asp 8–Arg 110

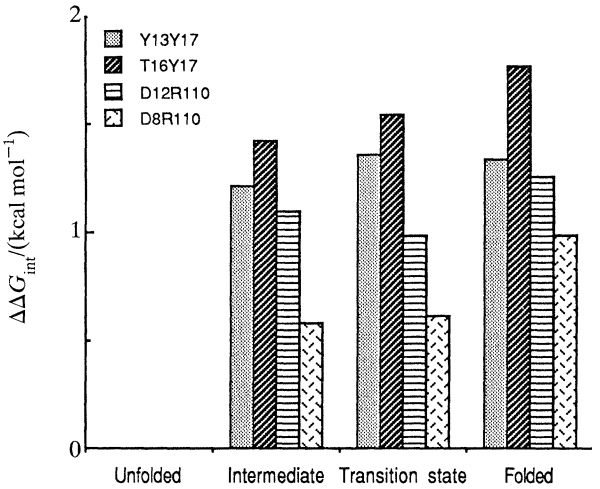

Figure 4. Difference energy diagrams for double mutant cycles (ϕ_{int}).

somewhat more so. This is further evidence that the N-terminus of the helix unwinds first during unfolding or forms late in folding.

7. DETECTION AND CHARACTERIZATION OF THE INTERMEDIATE BY NMR

Recently, NMR experiments has been used to detect folding intermediates. NMR can detect backbone > NH groups that undergo H/D exchange slowly with solvent because they are hydrogen-bonded within α-helices, β-sheets or other structural elements. Rapid quenching experiments on H/D exchange during refolding can detect secondary structure that is formed faster than is the final folded structure. For example, regions of α-helix and β-sheet are formed rapidly in the refolding of RNase A (Udgaonkar & Baldwin 1988) and in cytochrome *c* (Roder *et al.* 1988). More than 99% of the proton resonances in the ^{1}H-NMR spectrum of barnase have been assigned to their amino acid residues (Bycroft *et al.* 1990) and so we have used the H/D exchange procedure to detect a rapidly formed intermediate during refolding. Barnase was denatured and all exchangeable > NH groups deuterated (> 90% exchange) by incubating in 6.5 M (deuterated) urea and 99.8% D_2O at pD 6.3. The denatured and deuterated protein was allowed to refold by diluting into D_2O. Samples were taken during the refolding process and exposed to a labelling pulse of H_2O buffered at pH 8.5 where there is fast exchange of unprotected > ND deuterons. After sufficient time for complete refolding, the pH was lowered to 3.5 where exchange is very slow, and the fraction of H/D exchange measured by 2-D NMR. If folding is a simple one-step process, then the > ND deuterons at positions which undergo slow exchange in the folded protein should become protected from exchange concurrently with the overall process of refolding, which has a half life of 140 ms under these conditions in D_2O and 1.3 M urea. It is found, however, that several of these deuterons become protected with a half-life of about 12–30 ms. The rapidly protected positions are in the secondary structure of the two α-helices and the five strands of β-sheet. Three of the positions, the > ND(H)s of I25, N77 and S50, are protected according to a timecourse that is essentially identical to that of the overall refolding process. Significantly, I25, N77 and S50 make tertiary hydrogen bonds that are not within regular secondary structure but are a consequence of the overall fold of the molecule. This shows conclusively folding is multiphasic process in which an intermediate is rapidly formed. The data are consistent with the intermediate possessing some of the α and β secondary structure that is present in the final folded state.

8. DESCRIPTION OF INTERMEDIATE AND IMPLICATIONS

The NMR method gives information on just the secondary structure whereas protein engineering gives evidence, both quantitative and qualitative, on the interaction of side chains and indirect evidence about secondary structure. As well as the two procedures complementing each other, there is overlapping information that can be crosschecked. In every case, we find that there is complete agreement between NMR and protein engineering results. Both sets of experiments show that the transition state for unfolding and the folding intermediate have considerable secondary structure. The strands in the β-sheet are mainly intact, but loops are unfolded. The C-termini of the helices are relatively intact but the N-termini are unfolded. The hydrophobic core is weakened in the transition state for unfolding and even more so in the intermediate. One proposal for the prediction of the tertiary structure of proteins is to predict the repeating secondary structure, such as α-helices and β-sheets, which is less demanding than predicting tertiary structure, and then dock the helices and sheets. Our results suggest that docking of preformed elements is part of the rate determining process in folding and so this provides encouragement for that theoretical approach.

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