

# Early events in protein folding

Neil Ferguson and Alan R Fersht

Recent advances have significantly increased the time and spectroscopic resolution of protein folding experiments. We can now study the timescale and nature of polypeptide collapse, and how this correlates with secondary and tertiary structure formation. Studies on ultrafast folding proteins and peptides provide experimental benchmarks on a timescale that overlaps directly with that of molecular dynamics simulations. This makes possible direct tests of both simulations and current models of protein folding.

## Addresses

MRC Centre for Protein Engineering, MRC Centre, Hills Road, Cambridge CB2 2QH, UK

**Current Opinion in Structural Biology** 2003, 13:75–81

This review comes from a themed issue on  
Folding and binding  
Edited by Jane Clarke and Gideon Schreiber

0959-440X/03/\$ – see front matter  
© 2003 Elsevier Science Ltd. All rights reserved.

DOI 10.1016/S0959-440X(02)00009-X

## Abbreviations

**Csp** cold shock protein  
**SAXS** small-angle X-ray scattering  
**SH** Src homology

## Introduction

The traditional view of protein folding pathways has been that there is a preferred route to the native conformation via obligatory folding intermediates [1]. This dogma arose from the limited choice of proteins previously available for study and from the slow techniques that were typically employed for kinetic study. The past decade has seen the introduction of small recombinant proteins that fold by simple kinetics [2] and, over the past few years, the introduction of rapid reaction techniques, in particular temperature jump [3] and NMR line-broadening analysis [4–6], that can measure folding rates on both the sub-millisecond and submicrosecond timescales. There is a diverse array of proteins of differing topologies that typically fold rapidly and are well described by a two-state transition [2,7], whereby only the native and denatured ensembles are significantly populated in both equilibrium and kinetic experiments. These simple systems are also particularly amenable to ultrafast folding studies, as well as to the now standard procedure of analysing the structure of transition state ensembles using  $\Phi$ -values [8]. Some of these proteins unfold and fold so rapidly that their timescales overlap with that of *in silico* molecular

dynamics simulations performed at similar temperatures [4–6,9–13]. Here, we focus on critically reviewing recent experimental studies on the early events of protein folding reactions and their relevance to our current understanding of this complex process.

## Stages of protein folding

To simplify the discussion, we partition protein folding processes into the following stages, with the over-riding caveat that these processes need not be sequential or indeed temporally separated. First, specific or nonspecific chain collapse. Second, formation of secondary and tertiary structure, according to the balance of local and nonlocal, native and non-native interactions. Third, desolvation of the chain as it folds to lower energy conformations. The observed contribution of these events to protein folding and unfolding will depend on the protein under study, the chosen experimental conditions and perhaps the time regime of the experiment. Accordingly, it may be futile to search for a ‘universal’ folding mechanism and generalities of folding behaviour. It is perhaps more instructive to determine where on the continuum of behaviour a given protein lies and to see if homologous proteins behave similarly. But, by categorising folding events as described above, we can begin to see if there are underlying similarities or differences in the physics of protein folding for different mechanisms, topologies or timescales of conformational flux.

## Collapse in protein folding

The timescale of collapse and its correlation with the rate of secondary and tertiary structure formation have been key subjects of enquiry, as these phenomena are common to the folding of all proteins. Of particular interest are studies determining an upper limit for the rate of chain collapse [14], as this effectively establishes the maximum rate at which a given protein or element of structure can form in the absence of an energy barrier. If the maximum rate of collapse is determined, then the absolute height of energy barriers for folding and unfolding could be determined, rather than just comparing relative changes in activation energy, as is currently the case. (In the classical application of transition state theory, devised to describe covalent bond breakage and the formation of small molecules in the gas phase, the observed rate of a reaction is defined as  $k_{\text{obs}} = \kappa K_{\text{b}} T/h \cdot \exp(-E_{\text{a}}/RT)$ , where  $K_{\text{b}} T/h$  is a vibrational frequency of about  $10^{13} \text{ s}^{-1}$ . However, for complex reactions such as protein folding in aqueous solutions, whereby multiple noncovalent bonds are broken and formed in the transition state, it is clear that the ‘vibrational frequency’ will be slower and other effects, such as solvent viscosity and intramolecular viscosity, may

play a part. To this end, transition state theory has been modified to account for these effects [15]. There is still debate about the suitability of either approach to accurately model protein folding and unfolding reactions [16].) This may help us better understand the extent that different forces contribute to the free energy of different ensembles.

It is important to resolve the nature of polypeptide collapse, as well as its kinetics. Recently, Hagen and Eaton [17] have used nanosecond laser temperature-jump apparatus to monitor the collapse of cytochrome *c* from an extended ensemble to a compact denatured ensemble. Interestingly, the observed kinetics bear all the hallmarks of a two-state, barrier-limited process, namely single-exponential relaxation kinetics that exhibit Arrhenius law kinetics ( $\tau$  varies from approximately  $4.5\text{--}130 \times 10^{-5}$  s between 21 and 43°C). If this type of behaviour is typical of polypeptide collapse, it is a very significant finding. There is extensive debate on the specificity of polypeptide collapse [18,19], and whether it is a first-order transition with exponential kinetics or a continuous multiphase transition [20] with nonexponential kinetics. By determining the effects of solvent viscosity, temperature and chain composition on the kinetics of collapse and the ensemble free energy, and then resolving how these are temporally coupled with secondary and tertiary structure formation, we should be able to simulate folding more accurately and, therefore, understand protein folding better.

Kiefhaber and co-workers [21] examined the distance dependence of the rate of energy transfer between extrinsic dyes located at the chain termini of unstructured peptides; these chromophores were separated by varying numbers of intervening glycine and serine residues. There is an inverse linear relationship between the number of intervening peptide bonds and the rate of energy transfer, which is well described by a single-exponential function under all conditions. These results are again consistent with polypeptide collapse being barrier limited, with the fastest collapse observed at 22°C having a time constant of approximately 20 ns. Whereas the rate of collapse scales well with peptide length in these studies, the inverse relationship between the observed rate of collapse and solvent viscosity is somewhat less than predicted for reactions that are rate limited only by solvent friction [15]. This is interpreted to result from a combination of both solvent viscosity and internal (chain) viscosity, although there are uncertainties about the generalities of these findings because of the use of nonaqueous solvents and extrinsic labels, as well as the glycine-rich nature of the peptides.

Some of these issues are addressed by Eaton and co-workers [22], who probed intramolecular and intermolecular contact formation in peptides using intrinsic chro-

mophores in aqueous solutions. Under the experimental conditions used, intramolecular quenching of the optically excited triplet state of tryptophan by different quenching groups is significantly faster than competing intermolecular quenching. Furthermore, the distance dependence of the log rate of intramolecular quenching is almost linearly dependent on the log of the number of intervening peptide bonds ( $n$ ) and approximates to  $k \propto n^{-3/2}$ , except at low values of  $n$ . Interestingly, this relationship had been predicted by theoreticians using simplified models to describe the rate of end-to-end diffusive contact formation for polymers in solvent systems in which intrachain and chain-solvent interactions are balanced [23]. Subsequent experiments examined the effect of chain composition on the rate of end-to-end contact formation for helical and disordered peptides [24]. Surprisingly, the rate of end-to-end contact formation from the coil state is more rapid for helix-forming peptides than for peptides that are predicted to be intrinsically more dynamic or less ordered, although the rates of helix-coil relaxation are consistent with earlier measurements on other helical peptides [25–28]. The authors interpret these effects in terms of chain composition on excluded volume, backbone dynamics or hydrogen bonding, conclusions that are experimentally testable.

Small-angle X-ray scattering (SAXS) can also be used to probe polypeptide chain compaction. Time-resolved measurements are possible over a wide time range using stopped-flow [29] and micro-fabricated diffusional mixing devices [30], as well as pressure-jump apparatus [31]. Plaxco *et al.* [29] used stopped-flow SAXS to demonstrate that the 62-residue IgG-binding domain of protein L, which folds by a two-state transition, apparently folds directly to the native state in the absence of a fast collapse phase. Their experiments suggest that the radius of gyration of their particular denatured state does not change with denaturant concentration, which is crucial for their conclusion about the absence of a burst phase.

Whereas the refolding of staphylococcal nuclease is complex and multiphase in pH-jump and denaturant dilution studies [32,33], folding surprisingly appears to be mono-exponential in pressure-jump SAXS studies [31]. Similar refolding rates and activation volumes for the highly desolvated transition state were found using fluorescence, SAXS and Fourier transform IR spectroscopy [34] as structural probes, suggesting that collapse occurs concomitantly with secondary and tertiary structure formation. It is not clear why there are differences in the complexity of refolding of staphylococcal nuclease using different methods of perturbation, but perhaps the pressure-denatured state of this protein differs from the chemically denatured state. By contrast, the observed rate constants for the folding and unfolding of three cold shock protein (Csp) homologues, measured by stopped-flow and pressure jump, are the same within experimental error [35].

This suggests that the ground states in both pressure and chemical denaturation experiments are thermodynamically equivalent for these Csp homologues, although this may be protein dependent and must be experimentally verified rather than assumed. Thus, it is important to be cautious when interpreting results using just a single technique and, if possible, one should verify kinetic concordance using at least two or more independent techniques, especially when studying fast events. Site-directed mutants often provide excellent complementary controls, as mutations may produce changes in the kinetics and thermodynamics such that previously hidden behaviour may be characterised.

The apparent coupling of collapse and slow formation of the native state for staphylococcal nuclease and the IgG domain is perhaps a little surprising, given the earlier discussion about the rapidity of chain collapse. However, the fast collapse rates were measured from end-to-end contact formation rates either for smaller model peptides or for the denatured state of cytochrome *c*, which is not fully extended but cross-linked by its haem cofactor [17,21,22,36]. It is presently unclear just how representative these results are, how they scale with size, and whether concerted collapse and folding on a slower time-scale is more typical of larger polypeptides. However, it has been shown recently that chemical denaturants significantly decelerate intrachain contact formation in large polypeptides [37], such that collapse may become coupled to structure formation if there is a denaturant-dependent overlap of the time regimes of these processes. Alternatively, it is possible that the collapsed state of some proteins is less stable than the open denatured state and so collapse has to be coupled with subsequent folding. Interestingly, submillisecond time-resolved SAXS measurements show that cytochrome *c*, a protein almost twice the size of the IgG domain of protein L, does fold rapidly through a highly, collapsed non-native conformation in the microsecond time regime [30]. This suggests that sequence composition, topology and local structural propensity may augment or retard the rate of collapse, perhaps giving rise to the spectrum of folding behaviour experimentally observed.

### Secondary structure formation

The above studies are relevant to understanding not only global events such as chain collapse but also the formation of secondary structure and its subsequent coalescence. If an accurate length scaling of end-to-end contact formation rates and diffusion can be determined, then we have the beginnings of a quantitative answer to how secondary structure elements can form and collide during folding. Interestingly, it was demonstrated, in independent studies, that the diffusive rates of collision between reporter groups separated by 3–4 residues are very similar [21,22], with time constants ranging between 17 and 42 ns at 22°C. This time regime could be very significant for

secondary structure nucleation, as the number of residues necessary to form one turn of  $\alpha$  helix or the turn that connects a  $\beta$  hairpin (the smallest independently folded element of  $\beta$  structure) is also 3–4. From these studies, one may tentatively put an upper estimate on the folding time for helix or turn nucleation at 80–200 ns, although subsequent propagation of the *bona fide* element of structure may be substantially faster or slower. However, the rate constant of contact formation was measured for glycine-rich peptides [21,36] that *a priori* may be expected to have a greater flexibility than real protein sequences and thus the reported rates may be artificially fast. Further time-resolved studies on the effect of chain composition on the rates of contact formation and secondary structure formation are clearly required to test this.

The increased time resolution of laser temperature-jump experiments has allowed the fundamental reaction kinetics of helix-coil transitions to be characterised in some detail [24–28,38,39]. Whereas both the collapse and folding of some proteins can be adequately described by a two-state transition, the kinetics of  $\alpha$ -helix formation can be multiphasic [39–41], with both fast ( $\tau = 10$ –20 ns) and slow ( $\tau = 140$ –220 ns) relaxation phases. The observed relaxation kinetics of  $\alpha$ -helical peptides do not always fit to a single-exponential function and the folding of some helices may, therefore, be multistate.

Recent theoretical analyses of protein folding describe this process in terms of conformational diffusion along a multidimensional energy surface [42–44], on which parallel pathways to accessible free energy minima can exist. Such models predict a departure from single-exponential to stretched-exponential relaxation kinetics, as has been reported in several experimental studies [41,45,46]. But it is important to stress that the fast phase relaxation kinetics of  $\alpha$  helices are very close to the response times of the fastest contemporary laser temperature-jump instruments. Unfortunately, this current limitation introduces a degree of experimental uncertainty into measuring and, therefore, interpreting the reaction kinetics. This is especially true where kinetics are fitted to stretched-exponential functions, because the kinetic data may be fitted within experimental error to a series of sequential or parallel exponentials. Extreme caution must be exerted in interpreting such data. Furthermore, we occasionally observe nonexponential kinetics in our temperature-jump experiments that subsequently transpire to be exponential after artefacts have been eliminated. The early stages of temperature-jump experiments are highly susceptible to such artefacts.

Whereas the actual mechanism underlying helix-coil transitions in heteropeptides is still unclear, the emerging consensus is that the slower relaxation kinetics are caused by helix formation being rate limited by the formation of,

or flux through, a helix nucleation site [38,39]. The faster relaxation kinetics are attributed to rapid local dynamic events, such as helix fraying and rewinding, a process referred to as 'kinetic zippering' by Eaton and co-workers [39].

Jones and co-workers [47] observe helix formation that is, surprisingly, up to  $10^5$ -fold slower than the formation of the shorter helical peptides described above, with evidence of a helical overshoot in the reaction kinetics. This, however, may be an idiosyncratic result. The authors interpret their results in terms of 'slow' helix initiation rate limiting folding, with local helical propensity resulting in metastable, overlong helices that subsequently relax to an ensemble of shorter, more stable helices. Thus, very rapid and promiscuous propagation may indeed account for the helical overshoots observed in other proteins [48–51]. The existence of 'overwound' helices may have significant ramifications in proteins or peptides with high helical propensity, as metastable helical intermediates may form very rapidly during folding that are non-native in the sense that the register of the helices differs from that found in the native conformation.

A major problem in interpreting kinetics using some spectroscopic probes, such as the fluorescence of a particular tryptophan residue, is trying to understand how that particular probe reports the folding process. In studies of slower kinetics, where a highly cooperative transition is studied, individual probes generally report the whole process. But, in less cooperative transitions, individual probes may reflect just local events. For example, in time-resolved IR studies on the folding of the acid-destabilised core (E state) of apomyoglobin, Dyer and co-workers [52] found that folding kinetics probed by tryptophan fluorescence kinetics are mono-exponential, whereas multi-exponential kinetics are observed using IR spectroscopy. It was suggested that the multi-exponential kinetics reflect multiple pathways to the E state, a process hypothesized to occur as a consequence of conformational heterogeneity in the ground state ensembles. This example illustrates that, although it may be valid to follow the formation of the core by just using tryptophan fluorescence, it may be impossible to use this technique to probe conformations with substantial secondary structure but few tertiary interactions. In this respect, time-resolved IR absorbance studies show considerable promise as the technique of choice for future ultrarapid folding studies [25,40,45,53]. These techniques allow one to directly measure conformations that affect the stretching and vibration of amide C=O bonds and, by corollary, the time-dependent evolution of coil, helix, sheet and turn conformations. Understanding the kinetics of conformational changes is much more difficult and less direct when an intrinsic or extrinsic fluorophore is used as the spectroscopic probe.

Seminal work by Gai and co-workers [40,41] has taken these already challenging experiments to the next level of sophistication, by site-specifically labelling synthetic peptides using  $^{13}\text{C}=\text{O}$  instead of  $^{12}\text{C}=\text{O}$ . The real beauty of their experiments is in the selective isotopic labelling of each peptide, consequently introducing unique IR spectral characteristics that could be directly attributed to specific regions of the helix. Gai and co-workers used this strategy to measure the relaxation kinetics of both labelled and unlabelled regions of different labelled peptides in a single experiment. Selective labelling allows a direct comparison of the folding rates for different regions of the  $\alpha$ -helical peptide. Interestingly, whereas the N-terminal and central residues of the helical peptide fold at the same rates, the C-terminal residues unfold faster. Again, the observed kinetics can be fitted to either multi-exponential or stretched-exponential functions. These data suggest that, for the peptide studied, helix unfolding begins with the loss of one or more hydrogen bonds at the C terminus and it is possible that both the helical and coil conformations could be highly heterogeneous.

Helix formation is substantially faster than  $\beta$ -hairpin formation ( $\tau_{1/2} \approx 100\text{--}200$  ns [24–28,38,39] and 6  $\mu\text{s}$  [54], respectively) for the small number of peptides studied so far. In principle, if the formation of one turn (3–4 residues) is necessary and rate limiting in the folding of either an  $\alpha$  helix or a  $\beta$  hairpin, then these elements of structure could fold at similar rates. If, however, formation of a  $\beta$  hairpin is not initiated at the turn but by hydrophobic interactions between strands, then the increased chain entropy will impose energetic penalties that will most likely slow  $\beta$ -hairpin folding relative to the folding of helices. The strategy of using isotopically labelled polypeptides in time-resolved IR studies may make direct tests of folding mechanisms of peptides that form substructural elements feasible, for possibly the first time.

In, perhaps, the best-controlled study of the kinetic effects of viscosity reported so far, Hofrichter and co-workers [28] demonstrate that the folding rate constant of an  $\alpha$ -helical peptide is markedly less dependent on viscosity than that of a  $\beta$  hairpin, under conditions in which the equilibrium constant and activation energies are apparently unaffected by the viscogenic agent. The authors reconcile this effect (using Kramers theory [15]) in terms of differential effects of solvent viscosity on diffusive barrier crossing, with processes that have broad, low-energy barriers (here  $\beta$ -hairpin formation) being more susceptible to viscosity effects than reactions with sharper, higher energy barriers (here  $\alpha$ -helix folding). This interpretation, however, is just one of many possible and it is currently unclear whether the effects observed are general for these types of secondary structure or a consequence of the sequence composition. Until recently, it appeared that helical proteins fold much more rapidly than  $\beta$ -sheet proteins [2], possibly, as suggested

by Plaxco *et al.* [55], because of lower contact order in the helical proteins. However, the all- $\beta$ -sheet WW domains fold by two-state kinetics [10,11], with rate constants as high as those directly measured for the most rapidly folding  $\alpha$ -helical protein [12] and only marginally lower than those observed for  $\beta$ -hairpin formation [54]. As the formation of a  $\beta$  hairpin is rate limiting in the folding of one of the WW domains [10], a phenomenon predicted by molecular dynamics simulations to be rate limiting in another homologue [56], it may be that the folding of many all- $\beta$ -sheet proteins is initiated and rate limited by loop or hairpin nucleation, giving rise to structurally polarised transition states. Whereas further experiments will test the validity and generality of this hypothesis, comparative  $\Phi$ -value analyses of two homologous all- $\beta$ -sheet SH3 domains [57,58] appear to support this concept. It is clear, however, that both  $\beta$  sheets and multi-helix proteins can fold on similar timescales, although the dynamic range of folding times for these and mixed  $\alpha/\beta$  proteins spans many orders of magnitude [2].

### Formation of intermediates and tertiary structure

Unfortunately, the rate constants for folding of some of the ultrafast folding proteins have not been directly measured in the absence of denaturant or even at low denaturant concentrations [5,6,59,60], and the calculation of these rate constants has relied heavily on extrapolations to 0 M denaturant. There may be accumulation of folding intermediates with a consequent roll-over in the chevron plot [61] and so the observed rate constant may, in fact, contain terms for the final stages of folding in a multistate scheme. Many of the fast folding  $\alpha$ -helical proteins reported thus far, such as the  $\lambda$ -repressor [9], engrailed homeodomain [12], B domain of staphylococcal protein A [5] and the bacterial immunity proteins [62], have sequences with high intrinsic propensities to form secondary structure. Accordingly, these proteins may fold through highly helical intermediates or from highly structured ground states. As the rate constants for folding of several of these proteins have been determined using only NMR lineshape analysis, a procedure in which a two-state folding transition is assumed, it will be interesting to verify the relaxation rates using an independent technique and to establish the kinetic mechanism of folding.

Interestingly, the folding kinetics of Im7 and Im9, homologous immunity proteins with essentially identical folds, can be modelled to different kinetic schemes [62,63]. Im7, the protein with the higher intrinsic helical propensity, folds in an apparent three-state transition via a highly collapsed intermediate, whereas Im9 appears to fold in a two-state transition under similar conditions. In a technically demanding study, Capaldi *et al.* [63] used both continuous- and stopped-flow fluorescence apparatus to study the urea dependence of the folding and unfolding rate constants for Im7 on the submillisecond and milli-

second timescale. These authors fitted the observed transients for Im7 to a kinetic model in which folding occurs through an on-pathway intermediate. The subsequent  $\Phi$ -value analysis of Im7 [64] demonstrates that the intermediate contains substantial non-native hydrophobic interactions between three of its four  $\alpha$  helices. In this respect, it is interesting to note that the S6 ribosomal protein [65], a polypeptide that normally exhibits two-state folding, can be induced to misfold via a highly collapsed non-native conformation. The population of the misfolded intermediate of S6 was induced by the addition of high concentrations of sodium sulfate — which strengthens hydrophobic interactions — the same chemical used to stabilize the folding intermediate of Im7 [64]. Thus, it would appear that intermediates of the type described for Im7 and S6 are undoubtedly part of the energy landscape under defined solvent conditions, although some of these species may be described as mechanistically off-pathway in the sense that they have to unfold or rearrange before productive folding can occur. Many proteins do indeed fold by complex mechanisms or can be orchestrated to do so under appropriate conditions. But some kinetic phases that have previously been attributed to folding events have subsequently been demonstrated to be peripheral to folding [66,67]. Whether or not intermediates are on- or off-pathway is a debate that will run for years because it is so difficult to get definitive proof. In classical chemistry, an intermediate is considered to be on a reaction pathway if it is isolated and characterized, and it is formed and reacts fast enough to be on the pathway. Unfortunately, folding intermediates are rarely stable enough and sufficiently conformationally fixed to be isolated and characterized in suitable detail.

### Conclusions

We can now study fundamental processes of protein folding on a timescale that overlaps with that of computer simulation at the same temperature. Using measurements on a combination of model peptides, protein fragments and small domains, experimentalists can provide sufficient benchmarks to test the validity and details of molecular dynamics simulations and theoretical folding energy landscapes.

But these exciting possibilities bring a burden of responsibility for experimentalists and theoreticians. It is especially important that we benchmark simulations as rigorously as possible by experiment and learn from, as well as report, both our successes and failures. It is too easy to be tempted to glamorise inconclusive or preliminary experimental data by fitting it to results predicted from computer simulations. Experiments must be of sufficient resolution and accuracy to test theory rigorously. But there is no doubt that we have entered a new era in protein folding experiments that will contribute significantly to our understanding of this highly challenging biological problem.

## Acknowledgements

We would like to thank Valerie Daggett and members of the Fersht folding group for their helpful discussions and critical reading of this manuscript.

## References

- Baldwin RL: **Protein chemistry - Pieces of the folding puzzle.** *Nature* 1990, **346**:409-410.
- Jackson SE: **How do small single-domain proteins fold?** *Fold Des* 1998, **3**:R81-R91.
- Nolting B, Golbik R, Fersht AR: **Submillisecond events in protein-folding.** *Proc Natl Acad Sci USA* 1995, **92**:10668-10672.
- Huang GS, Oas TG: **Submillisecond folding of monomeric lambda-repressor.** *Proc Natl Acad Sci USA* 1995, **92**:6878-6882.
- Myers JK, Oas TG: **Preorganized secondary structure as an important determinant of fast protein folding.** *Nat Struct Biol* 2001, **8**:552-558.
- Spector S, Raleigh DP: **Submillisecond folding of the peripheral subunit-binding domain.** *J Mol Biol* 1999, **293**:763-768.
- Fersht A: *Structure And Mechanism In Protein Science: A Guide To Enzyme Catalysis And Folding.* New York: Freeman; 1999.
- Fersht AR, Matouschek A, Serrano L: **The folding of an enzyme. 1. Theory of protein engineering analysis of stability and pathway of protein folding.** *J Mol Biol* 1992, **224**:771-782.
- Burton RE, Huang GS, Daugherty MA, Fullbright PW, Oas TG: **Microsecond protein folding through a compact transition state.** *J Mol Biol* 1996, **263**:311-322.
- Jager M, Nguyen H, Crane JC, Kelly JW, Gruebele M: **The folding mechanism of a  $\beta$ -sheet: the WW domain.** *J Mol Biol* 2001, **311**:373-393.
- Ferguson N, Johnson CM, Macias M, Oschkinat H, Fersht AR: **Ultrafast folding of WW domains without structured aromatic clusters in the denatured state.** *Proc Natl Acad Sci USA* 2001, **98**:13002-13007.
- Mayor U, Johnson CM, Daggett V, Fersht AR: **Protein folding and unfolding in microseconds to nanoseconds by experiment and simulation.** *Proc Natl Acad Sci USA* 2000, **97**:13518-13522.
- Kuhlman B, Evans PA, Raleigh DP: **Submillisecond folding of the N-terminal domain of the ribosomal protein L9.** *Biophys J* 1998, **74**:A128.
- Hagen SJ, Hofrichter J, Eaton WA: **Rate of intrachain diffusion of unfolded cytochrome c.** *J Phys Chem B* 1997, **101**:2352-2365.
- Kramers HA: *Physica* 1940, **4**:283-304.
- Jacob M, Schmid FX: **Protein folding as a diffusional process.** *Biochemistry* 1999, **38**:13773-13779.
- Hagen SJ, Eaton WA: **Two-state expansion and collapse of a polypeptide.** *J Mol Biol* 2000, **301**:1019-1027.
- Qi PX, Sosnick TR, Englander SW: **The burst phase in ribonuclease A folding and solvent dependence of the unfolded state.** *Nat Struct Biol* 1998, **5**:882-884.
- Sosnick TR, Shtilerman MD, Mayne L, Englander SW: **Ultrafast signals in protein folding and the polypeptide contracted state.** *Proc Natl Acad Sci USA* 1997, **94**:8545-8550.
- Chan HS, Dill KA: **Polymer principles in protein-structure and stability.** *Annu Rev Biophys Biophys Chem* 1991, **20**:447-490.
- Bieri O, Wirz J, Hellrung B, Schutkowski M, Drewello M, Kiefhaber T: **The speed limit for protein folding measured by triplet-triplet energy transfer.** *Proc Natl Acad Sci USA* 1999, **96**:9597-9601.
- Lapidus LJ, Eaton WA, Hofrichter J: **Measuring the rate of intramolecular contact formation in polypeptides.** *Biophys J* 2001, **80**:721.
- Szabo A, Schulten K, Schulten Z: **First passage time approach to diffusion controlled reactions.** *J Chem Phys* 1980, **72**:4350-4357.
- Lapidus LJ, Eaton WA, Hofrichter J: **Measuring dynamic flexibility of the coil state of a helix-forming peptide.** *J Mol Biol* 2002, **319**:19-25.
- Huang CY, Klemke JW, Getahun Z, DeGrado WF, Gai F: **Temperature-dependent helix-coil transition of an alanine based peptide.** *J Am Chem Soc* 2001, **123**:9235-9238.
- Lednev IK, Karnoup AS, Sparrow MC, Asher SA: **Alpha-helix peptide folding and unfolding activation barriers: a nanosecond UV resonance Raman study.** *J Am Chem Soc* 1999, **121**:8074-8086.
- Lednev IK, Karnoup AS, Sparrow MC, Asher SA: **Transient UV Raman spectroscopy finds no crossing barrier between the peptide alpha-helix and fully random coil conformation.** *J Am Chem Soc* 2001, **123**:2388-2392.
- Jas GS, Eaton WA, Hofrichter J: **Effect of viscosity on the kinetics of alpha-helix and beta-hairpin formation.** *J Phys Chem B* 2001, **105**:261-272.
- Plaxco KW, Millett IS, Segel DJ, Doniach S, Baker D: **Chain collapse can occur concomitantly with the rate-limiting step in protein folding.** *Nat Struct Biol* 1999, **6**:554-556.
- Pollack L, Tate MW, Darnton NC, Knight JB, Gruner SM, Eaton WA, Austin RH: **Compactness of the denatured state of a fast-folding protein measured by submillisecond small-angle x-ray scattering.** *Proc Natl Acad Sci USA* 1999, **96**:10115-10117.
- Woenckhaus J, Kohling R, Thiyagarajan P, Littrell KC, Seifert S, Royer CA, Winter R: **Pressure-jump small-angle x-ray scattering detected kinetics of staphylococcal nuclease folding.** *Biophys J* 2001, **80**:1518-1523.
- Chen HM, Markin VS, Tsong TY: **pH-induced folding/unfolding of staphylococcal nuclease - determination of kinetic-parameters by the sequential-jump method.** *Biochemistry* 1992, **31**:1483-1491.
- Wallenhorst WF, Green SM, Roder H: **Kinetic evidence for folding and unfolding intermediates in staphylococcal nuclease.** *Biochemistry* 1997, **36**:5795-5805.
- Panick G, Malessa R, Winter R, Rapp G, Frye KJ, Royer CA: **Structural characterization of the pressure-denatured state and unfolding/refolding kinetics of staphylococcal nuclease by synchrotron small-angle X-ray scattering and Fourier-transform infrared spectroscopy.** *J Mol Biol* 1998, **275**:389-402.
- Perl D, Holtermann G, Schmid FX: **Role of the chain termini for the folding transition state of the cold shock protein.** *Biochemistry* 2001, **40**:15501-15511.
- Lapidus LJ, Eaton WA, Hofrichter J: **Measuring the rate of intramolecular contact formation in polypeptides.** *Proc Natl Acad Sci USA* 2000, **97**:7220-7225.
- Hagen SJ, Carswell CW, Sjolander EM: **Rate of intrachain contact formation in an unfolded protein: temperature and denaturant effects.** *J Mol Biol* 2001, **305**:1161-1171.
- Thompson PA, Munoz V, Jas GS, Henry ER, Eaton WA, Hofrichter J: **The helix-coil kinetics of a heteropeptide.** *J Phys Chem B* 2000, **104**:378-389.
- Thompson PA, Eaton WA, Hofrichter J: **Laser temperature jump study of the helix-coil kinetics of an alanine peptide interpreted with a 'kinetic zipper' model.** *Biochemistry* 1997, **36**:9200-9210.
- Huang CY, Getahun Z, Wang T, DeGrado WF, Gai F: **Time-resolved infrared study of the helix-coil transition using C-13-labeled helical peptides.** *J Am Chem Soc* 2001, **123**:12111-12112.
- Huang CY, Getahun Z, Zhu YJ, Klemke JW, DeGrado WF, Gai F: **Helix formation via conformation diffusion search.** *J Am Chem Soc* 2002, **99**:2788-2793.
- Bryngelson JD, Onuchic JN, Socci ND, Wolynes PG: **Funnels, pathways, and the energy landscape of protein-folding - a synthesis.** *Proteins* 1995, **21**:167-195.
- Hummer G, Garcia AE, Garde S: **Helix nucleation kinetics from molecular simulations in explicit solvent.** *Proteins* 2001, **42**:77-84.

44. Hummer G, Garcia AE, Garde S: **Conformational diffusion and helix formation kinetics.** *Phys Rev Lett* 2000, **85**:2637-2640.
45. Leeson DT, Gai F, Rodriguez HM, Gregoret LM, Dyer RB: **Protein folding and unfolding on a complex energy landscape.** *Proc Natl Acad Sci USA* 2000, **97**:2527-2532.
46. Sabelko J, Ervin J, Gruebele M: **Observation of strange kinetics in protein folding.** *Proc Natl Acad Sci USA* 1999, **96**:6031-6036.
47. Clarke DT, Doig AJ, Stapley BJ, Jones GR: **The alpha-helix folds on the millisecond time scale.** *Proc Natl Acad Sci USA* 1999, **96**:7232-7237.
48. Kuwajima K, Yamaya H, Sugai S: **The burst-phase intermediate in the refolding of  $\beta$ -lactoglobulin studied by stopped-flow circular dichroism and absorption spectroscopy.** *J Mol Biol* 1996, **264**:806-822.
49. Forge V, Hoshino M, Kuwata K, Arai M, Kuwajima K, Batt CA, Goto Y: **Is folding of beta-lactoglobulin non-hierarchical? Intermediate with native-like  $\beta$ -sheet and non-native alpha-helix.** *J Mol Biol* 2000, **296**:1039-1051.
50. Kuwata K, Shastry R, Cheng H, Hoshino M, Batt CA, Goto Y, Roder H: **Structural and kinetic characterization of early folding events in beta-lactoglobulin.** *Nat Struct Biol* 2001, **8**:151-155.
51. Shastry RMC, Roder H, Kuwata K, Goto Y: **Kinetic evidence for non-native A-helix at the N-terminus of  $\beta$ -lactoglobulin.** *Biophys J* 2001, **80**:722.
52. Gulotta M, Gilmanishin R, Buscher TC, Callender RH, Dyer RB: **Core formation in apomyoglobin: probing the upper reaches of the folding energy landscape.** *Biochemistry* 2001, **40**:5137-5143.
53. Colley CS, Clark IP, Griffiths-Jones SR, George MW, Searle MS: **Steady state and time-resolved IR spectroscopy of the native and unfolded states of bovine ubiquitin: protein stability and temperature-jump kinetic measurements of protein folding at low pH.** *Chem Commun* 2000, **16**:1493-1494.
54. Munoz V, Thompson PA, Hofrichter J, Eaton WA: **Folding dynamics and mechanism of  $\beta$ -hairpin formation.** *Nature* 1997, **390**:196-199.
55. Plaxco KW, Simons KT, Baker D: **Contact order, transition state placement and the refolding rates of single domain proteins.** *J Mol Biol* 1998, **277**:985-994.
56. Ferguson N, Pires JR, Toepert F, Johnson CM, Pan YP, Daggett V, Oschkinat H, Fersht AR: **Using flexible loop mimetics to extend Phi-value analysis to secondary structure interactions.** *Proc Natl Acad Sci USA* 2001, **98**:13008-13013.
57. Martinez JC, Serrano L: **The folding transition state between SH3 domains is conformationally restricted and evolutionarily conserved.** *Nat Struct Biol* 1999, **6**:1010-1016.
58. Riddle DS, Grantcharova VP, Santiago JV, Alm E, Ruczinski I, Baker D: **Experiment and theory highlight role of native state topology in SH3 folding.** *Nat Struct Biol* 1999, **6**:1016-1024.
59. Lee JC, Gray HB, Winkler JR: **Cytochrome c' folding triggered by electron transfer: fast and slow formation of four-helix bundles.** *Proc Natl Acad Sci USA* 2001, **98**:7760-7764.
60. Burton RS, Myers JK, Oas TG: **Ultrafast folding of monomeric  $\lambda$ -repressor: experimental characterization and accurate theoretical prediction of folding rates.** *Biophys J* 1998, **74**:A24.
61. Roder H, Colon W: **Kinetic role of early intermediates in protein folding.** *Curr Opin Struct Biol* 1997, **7**:15-28.
62. Ferguson N, Capaldi AP, James R, Kleanthous C, Radford SE: **Rapid folding with and without populated intermediates in the homologous four-helix proteins Im7 and Im9.** *J Mol Biol* 1999, **286**:1597-1608.
63. Capaldi AP, Shastry MCR, Kleanthous C, Roder H, Radford SE: **Ultrarapid mixing experiments reveal that Im7 folds via an on-pathway intermediate.** *Nat Struct Biol* 2001, **8**:68-72.
64. Capaldi AP, Kleanthous C, Radford SE: **Im7 folding mechanism: misfolding on a path to the native state.** *Nat Struct Biol* 2002, **9**:209-216.
65. Otzen DE, Oliveberg M: **Salt-induced detour through compact regions of the protein folding landscape.** *Proc Natl Acad Sci USA* 1999, **96**:11746-11751.
66. Arcovito A, Gianni S, Brunori M, Travaglini-Allocatelli C, Bellelli A: **Fast coordination changes in cytochrome c do not necessarily imply folding.** *J Biol Chem* 2001, **276**:41073-41078.
67. Jones CM, Henry ER, Hu Y, Chan CK, Luck SD, Bhuyan A, Roder H, Hofrichter J, Eaton WA: **Fast events in protein-folding initiated by nanosecond laser photolysis.** *Proc Natl Acad Sci USA* 1993, **90**:11860-11864.