Time-resolved biophysical methods in the study of protein folding

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Many of the biophysical techniques developed to characterize native proteins at equilibrium have now been adapted to the structural and thermodynamic characterization of transient intermediate populations during protein folding. Recent advances in these techniques, the use of novel methods of initiating refolding, and a convergence of theoretical and experimental approaches are leading to a detailed understanding of many aspects of the folding process.

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Abbreviations

ANS	8-anilino-1-naphthalenesulphonate
CI2	chymotrypsin inhibitor 2
MS	mass spectrometry
NOE	nuclear Overhauser effect
SAXS	small-angle X-ray scattering
T-jump	temperature jump

Introduction

The primary question addressed in studies of protein folding can be stated very simply: how do denatured polypeptide chains limit their conformational search in order to achieve the native state in a biologically relevant time? The complexity of the denatured state rules out the possibility that folding is a simple stochastic search process [1], and folding is, almost certainly, facilitated by the existence of (potentially multiple) specific mechanisms. Much effort has gone into the characterization of transient partially folded states arising during folding [2,3•] in an attempt to understand these mechanisms and the process by which proteins rapidly fold to their native structures.

Native proteins are characterized by a high degree of compactness, an ordered hydrophobic core, a well defined overall architecture, and the presence of specific and cooperative interactions among buried side chains. Recent progress in both instrumentation and experimental design has provided unprecedented insights into the evolution of each of these characteristics as an initially disordered and extended polypeptide chain folds via a heterogeneous population of partially folded states into its native conformation (Fig. 1). In this article we focus on recent advances in the time-resolved experimental characterization of the properties and distribution of partially folded states arising during nonoxidative refolding *in vitro*, and the promise that these developments hold for providing a detailed description of the folding process.

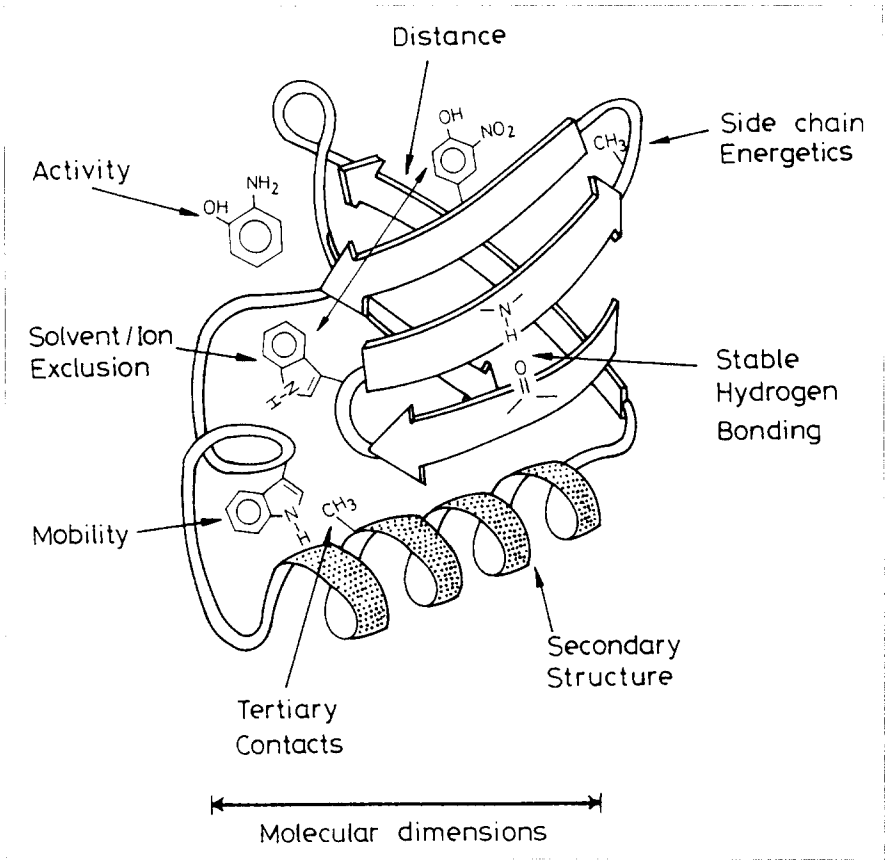
The initiation of folding

Protein folding in the cell follows synthesis of the polypeptide chain on a ribosome. Refolding *in vitro* is more readily initiated by rapidly transferring a protein from denaturing conditions to an environment in which the native conformation is favoured. This is often achieved by diluting protein solutions containing denaturant with nondenaturing buffers using a stopped-flow mixing device. Turbulent mixers, such as the Berger ball mixer used in many commercially available instruments, achieve high mixing efficiency by interweaving fine, turbulence-generated streams [4]. The minimum dimensions of these streams is limited by technical issues, such as cavitation, such that denaturants require $>100\,\mu s$ to diffuse from them. Limits on the physical proximity of a detecting cell to the mixer, and the speed with which flow can be stopped without producing shock effects, further increase the deadtime of most stopped-flow instruments to >1 ms.

The extremely rapid burst-phase events now evident for many proteins are complete within the deadtime of conventional stopped-flow mixing devices [5]. Fortunately, recent technical advances promise significant reductions in these mixing deadtimes. The use of continuous-flow devices that avoid the shock disturbances of high-speed stopped-flow and 'freejet' mixers, which generate small, rapidly diffusing streams by laminar flow through very small orifices, has lowered deadtimes to tens of microseconds [6•]. Non-mixing methods, such as flash photolysis [7], optical electron transfer [8•] and temperature jump (T-jump) [9•,10•], promise further improvements. Optical electron transfer, based on the existence of conditions under which an oxidized redox protein is unfolded but the reduced form is native, has been used to initiate the refolding of cytochrome *c* in $<1\,\mu s$ by photochemically induced reduction [8•]. T-jump experiments, based on reversing cold-induced denaturation through rapid sample heating, have yielded deadtimes of $\sim 10\,\mu s$ by electrical-discharge heating [9•] and an amazing ~ 20 ns by laser-induced heating [10•]. Applied to the folding of apomyoglobin, laser T-jump has been used to characterize a collapsed state formed in a diffusion-limited reaction that is completed within $\sim 20\,\mu s$ [10•]. When coupled with high-speed absorbance, fluorescence and CD, these new folding-initiation techniques will undoubtedly provide important insights into the chemistry of the earliest events in folding.

Figure 1

A schematic representation of the characteristics of globular proteins that can be followed during refolding, with time resolution in the second to millisecond range. Other properties that can be monitored, but are not indicated, include the creation and disruption of organized hydrophobic voids and overall thermodynamic stability. Although no individual probe can monitor all of the structural details of a folding intermediate, the use of multiple complementary probes can provide a detailed picture of the distribution of conformations that make up transient folding populations.



Measuring collapse and core packing

A general property of protein folding is that an extended and highly disordered polymer chain must collapse to form a compact, globular protein [11•]. Measures of molecular dimensions and core packing (Table 1) are thus critical elements of a complete description of the folding process. Indirect probes of these properties, such as changes in the UV absorbance of aromatic residues [12•], the fluorescence of tryptophan or tyrosine side chains [13••], or the fluorescence of extrinsic fluorophores such as 8-anilino-1-naphthalenesulphonate (ANS) [14•], have seen widespread application. More direct probes of the exclusion of solvent from the hydrophobic core, involving monitoring the accessibility of hydrophilic fluorescence quenchers such as iodide or acrylamide [13••] or the reactivity of cysteine side chains [15], have also been developed. The use of time-resolved fluorescence spectroscopy, not only to monitor molecular dimensions but also to provide a detailed description of the loss of core residue mobility during the refolding of dihydrofolate reductase [16•], is a recent example of the variety of indirect indicators of the collapse and core packing that are available. What most of these probes lack, however, is an ability to monitor the distribution of individual species in heterogeneous mixtures or to provide a quantitative measure of the dimensions of partially folded conformations. Although little progress has been made on the former, several quantitative probes of molecular dimension are now available.

Time-resolved fluorescence energy transfer, small-angle X-ray scattering (SAXS), and quasi-elastic light scattering have all been used to provide a direct measurement of the dimensions of species arising during folding. The detection of fluorescence energy transfer between a covalently attached fluorophore and a tryptophan side chain, which has been used to attempt direct measurements of the evolution of collapsed species during the refolding of apomyoglobin [17•] and other proteins [18], is consistent with the hypothesis that these proteins fold via a rapidly formed intermediate of near-native compactness. Such studies are, however, limited to proteins that can be modified with suitable fluorophores and only provide measurements of a single scalar distance. Unlike fluorescence energy transfer, SAXS [19] and quasi-elastic light scattering provide direct means of monitoring the overall dimensions of macromolecules. SAXS, when implemented with very high flux synchrotron X-ray sources, provides a measure of the average radius of gyration with < 100 ms time resolution. This technique has recently been applied to the refolding of apomyoglobin, again indicating the near-native compactness of the major folding intermediate of this protein [20•,21]. Quasi-elastic light scattering, though presently limited by a ~ 1 s deadtime, monitors

Table 1

Biophysical techniques used to investigate protein folding*.

Property	Technique	Resolution	Measurement	Reference
Core packing	Intrinsic fluorescence	< 1 ms	The orientation and environment of (predominantly) tryptophan side chains	[13••]
	Ultraviolet absorbance	ms	The orientation and environment of (predominantly) tyrosine side chains	[12•]
	Extrinsic (ANS) fluorescence	ms	Formation and disruption of organized hydrophobic patches and clefts	[14•]
	Fluorescence quenching	ms	Isolation of tryptophan side chains from hydrophilic fluorescence quenchers	[13••]
	Cysteinyl quenching	10 s	Protection of cysteine side chains from hydrophilic reactants	[15]
Molecular dimensions	Fluorescence anisotropy	ms	Tryptophan side chain mobility and overall molecular dimensions	[16•]
	Fluorescence energy transfer	ms	Scalar distance between tryptophan and a covalently attached fluorophore	[17•]
	Small angle X-ray scattering	< 100 ms	The average radius of gyration	[20•]
	Quasi-elastic light scattering	1 s	The average radius of gyration	[22]
Secondary structure and persistent hydrogen bonds	Far-UV circular dichroism	ms	Backbone conformation averaged over sequence and population	[5]
	Pulse labelling NMR	5–10 ms	Sequence specific formation of stable amide and tryptophan hydrogen bonds	[24]
	Pulse labelling mass spectrometry	5–10 ms	The formation of persistent hydrogen bonds in discrete intermediates	[25•]
Tertiary contacts and native structure	Biological activity	ms–s	The formation of native tertiary structure at the active site	[5]
	Interrupted folding	10 ms	The unfolding rate of discrete intermediates as a probe of their stability	[30]
	Near-UV circular dichroism	ms	Formation of stable aromatic and disulphide bond tertiary contacts	[5]
	Real-time NMR	1 s	Formation of specific side chain tertiary contacts	[35••]
	Protein engineering	†	The energetic contributions of side chains to discrete intermediates	[32••]

*Many of the biophysical techniques developed to characterize native proteins at equilibrium have now been adapted to the structural and thermodynamic characterization of transient populations during folding. Here we summarize many of the biophysical techniques that have been used in recent years to characterize the folding of a variety of proteins. A single reference to each method is provided that either reflects a recent review of the subject or an illustrative application of the technique. †The time resolution of protein-engineering refolding experiments is limited only by the time resolution of the probe used to monitor folding mutants.

the translational mobility and thus overall dimensions of a macromolecule, and has been used to probe the formation of compact states during the refolding of lysozyme [22]. There appears to be no fundamental reason why these techniques will not prove to be general methods for observing directly the dimensions of a polypeptide chain during protein folding.

Monitoring the formation of secondary structure

Probes of the backbone conformation, such as far-UV CD and pulse-labelling hydrogen exchange, have provided a wealth of data on the kinetics of secondary-structure formation during folding (Table 1). The recovery of far-UV CD ellipticity is widely considered a critical measure of the average secondary structure content in heterogeneous folding mixtures. For proteins, however, much of the formation of secondary structure occurs in a burst phase during the mixing deadtime and thus has not been amenable to direct study. It is fortunate, then, that the invention of extremely rapid methods for the initiation of refolding comes close on the heels of advances in high-speed CD [23]. Now that the application of high-intensity laser light sources to CD spectropolarimetry has produced sub-μs time resolution, fundamental questions about the timing of the formation of secondary structure may soon be answered.

Although CD provides an estimate of average secondary structure content, it does not provide information on the specific residues involved or the distribution of conformations present. Pulse-labelling amide-exchange experiments can provide this complementary information by monitoring the formation of stable backbone hydrogen bonds [24]. Pulse labelling linked to NMR spectroscopy has been used for a number of years as a probe of the sequence-specific formation of persistent elements of secondary structure but, like optical methods, the technique cannot resolve individual components from heterogeneous mixtures. Advances in coupling pulse

labelling and mass spectrometry (MS) have furthered our understanding of the formation of secondary structure by allowing the observation of resolved molecular species. This has provided a means of characterizing the hydrogen-exchange properties of discrete species in heterogeneous populations, as observed, for example, during the refolding of lysozyme [25•]. MS, like optical methods, provides data averaged over the entire sequence of a molecule. Technical advances in MS, however, have proven the feasibility of identifying the sequences of protein cleavage products produced in the gas phase by collision-induced dissociation [26,27•]. It may thus soon prove possible to produce sequence-specific hydrogen-exchange data for discrete species in complex folding populations.

Detecting tertiary contacts

Because the formation of partially ordered states with regions of native-like structure is thought to be an essential step in protein folding, detecting native tertiary contacts in transient folding populations has been a major goal of folding research. Near-UV CD, which primarily monitors the aromatic side chains immobilized by asymmetric tertiary contacts, has proved an important probe of the recovery of native structure [5]. For many proteins, time-resolved assays of the recovery of biological activity (e.g. the binding of fluorescent substrates or inhibitors) can be used to monitor the recovery of a native active site [12•,28]. Interrupted folding experiments, in which transiently refolded mixtures undergo a second unfolding by the rapid addition of denaturant, have been used to detect the formation of material with native stability [29]. This method, which relies on the reasonable assumption that the unfolding rate of a given conformation reflects its thermodynamic stability, has recently been used to monitor the stability of an intermediate in the folding of barnase [30] and to support the existence of parallel pathways in the folding of lysozyme [31•]. It may provide a general probe of the formation of both native and near-native structures.

Although probes of the formation of native protein are well established, only recently have techniques been developed that can monitor the formation of specific tertiary contacts during folding. Protein engineering provides one method of assaying the contributions of specific side chains to the energetics of transient folding intermediates. The contributions of these side chains (relative to their contribution to the stability of the native protein) have been interpreted as a measure of the 'nativeness' of their contacts in the intermediate. Major folding intermediates of barnase [32••] and phosphoglycerate kinase [33•] have been characterized using this technique. Stopped-flow NMR has also been used to monitor the formation of specific tertiary interactions during folding [34,35••]. Though presently limited to a time resolution of ~1 s, the technique provides a nonperturbing method of detecting the formation of the highly shifted resonances characteristic of native proteins. This method shows much potential for providing information concerning the formation of specific native and native-like contacts during folding.

Transition-state probes

A complete description of the folding process requires knowledge of both the structure and energetics of the rate-determining conformation. While the ephemeral nature of transition states generally precludes direct structural studies, the transition state is the conformation of the rate-limiting step (or steps) and therefore the kinetics of folding can provide an indirect probe of its structure. The effect of environmental factors and mutations on the kinetics of the recovery of native properties (such as fluorescence) have thus been used to provide a detailed picture of the conformation of this most fleetingly transient species in protein folding.

Environmental factors that affect folding rates have provided valuable clues to the general nature of folding transition states. For example, studies of the temperature dependence of protein folding rates have been used to probe their thermodynamic properties [36•]. Other studies into the effects of pressure [37•], denaturants [38], ionic strength [39] and pH [40], have been used to define relative molar volumes and solvent-exposed surface areas of transition states, and to probe the contributions of ionizable groups to their energetics. From such studies, a general picture is emerging of a typical transition state as a collapsed but still relatively poorly packed set of conformations.

Efforts to ascertain the high-resolution structure of a folding transition state have focused on protein engineering experiments designed to produce a map of the energetic contributions of specific side chains to the rate-limiting step. This has been carried out in some detail for barnase and chymotrypsin inhibitor 2 (CI2) [32••] in studies that have provided insights into the structure and heterogeneity of the transition state [41], and suggest that small nuclei of native-like structure are involved in the rate-determining steps of the folding of at least some proteins.

Conclusions

As the number and quality of biophysical techniques with sufficient time resolution increases, so our detailed knowledge of the folding process improves. Issues such as cooperativity, collapse and the formation of secondary structure during refolding are becoming well described for a number of proteins. What is still lacking, however, is a means of generating a picture of the structure and distributions of transient folding populations with good spatial resolution. The next challenge in protein folding lies in discovering how to produce such high-resolution data. Several potential approaches now appear feasible.

Because no single method can provide a complete picture of the distribution of structures in a transient population of intermediates (Fig. 1), it is clear that multiple complementary approaches must be combined to generate detailed structural models. For example, dynamic light-scattering and intrinsic fluorescence can be used to define the average dimensions and degree of core packing in a population, pulse-labelling amide exchange can provide information on the location and stability of secondary structure, and NMR and inhibitor binding can be used to define specific tertiary contacts. Such information can thus be brought together to develop a detailed picture of the key features the folding process [12•,42••,43•].

The direct acquisition of high-resolution structural information may also be possible through modifications of current biophysical methods. NMR, for example, was converted from a technique of low spatial resolution to one applicable to high spatial resolution by the introduction of two-dimensional spectroscopy. The application of multi-dimensional NMR techniques to the study of highly transient structures may appear daunting, but the increased availability of specifically isotopically labeled proteins and high-field spectrometers has already made possible two-dimensional refolding experiments with a time resolution of a few minutes [44•] by repetitive accumulation of rapidly acquired spectra. Novel approaches may provide significant further reductions. For example, the refolding of a protein during the acquisition of two-dimensional data can result in changes in peak shape that can be deconvoluted to provide a wealth of information on the refolding kinetics of individual elements of the protein with time resolution on the order of seconds. Further potential exists for experiments in which nuclear Overhauser effect (NOE) crosspeaks generated in folding populations are detected in the spectrally well characterized native protein, possibly to provide a detailed picture of the tertiary contacts formed in transient folding intermediates (J Balbach *et al.*, unpublished data).

Theoretical methods can provide atomic-level models of the structure and distribution of protein folding intermediates but they necessarily involve significant simplifying assumptions. The iterative coupling of simulation with experiment may provide the necessary constraints on these assumptions to produce accurate high-resolution models. Recent studies of the details of denatured protein conformations have provided an example of this type of complementary theoretical and experimental approach. Current understanding of the 'random coil' denatured state has recently been advanced by the use of experiments to verify the specific predictions produced from Monte Carlo simulations of the denatured state. Further refinements of these simulations based on discrepancies between predicted and observed NOEs and J-coupling constants [45••] has led to a deeper understanding of the conformational distributions within denatured states.

A similar combined simulation and experimental approach has been used for the interpretation of protein engineering investigations into the structure of the folding transition states of CI2 and barnase. Molecular dynamics simulations, here inspired and constrained by experimental investigations, proved vital for the formation of high-resolution models of these folding transition states [32••,46••,47••]. The complementary aspects of theory and experiment in protein folding suggest that this promising trend will continue. In particular, it is to be hoped that, with the addition of experimentally derived constraints, lattice simulations [47••,48•,49•] will lead to higher-resolution models of intermediate populations with significant predictive value. The use of complementary biophysical approaches to obtain adequate information to constrain theoretical models holds great promise for providing a detailed description and understanding of the folding process.

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