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The folding process of hen lysozyme: a perspective from the 'new view'

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Abstract. How a conformationally disordered polypeptide chain rapidly and efficiently achieves its well-defined native structure is still a major question in modern structural biology. Although much progress has been made towards rationalizing the principles of protein structure and dynamics, the mechanism of the folding process and the determinants of the final fold are not yet known in any detail. One protein for which folding has been studied in great detail by a combination of diverse techniques is hen lysozyme. In this article we review the present state of our knowledge of the folding process of this enzyme and focus in particular on recent experiments to probe some of its specific features. These results are then discussed in the context of the 'new view' of protein folding based on energy surfaces and landscapes. It is shown that a schematic energy surface for lysozyme folding, which is broadly consistent with our experimental data, begins to provide a unified model for protein folding through which experimental and theoretical ideas can be brought together.

Key words. Protein folding; hen egg white lysozyme; biophysical methods; kinetic partitioning; folding intermediates; folding domains; nonnative interactions; energy surface.

Introduction

While it is widely accepted that the amino acid sequence contains all the information required to achieve the native molecular architecture of at least most proteins [1], the detailed process by which an unfolded polypeptide chain achieves its specific three-dimensional (3D) structure is not yet understood. Unravelling the mechanism of protein folding would constitute a major advance in our understanding of the manner in which the genetic code is translated into biological activity. It would also provide a basis for the rational modification and design of proteins and help in the prediction of their folds from amino acid sequences. The fact that folding occurs many orders of magnitude more rapidly than predicted by a random search of conformational space [2, 3] indicates that the nature of the interactions that develop within a protein structure serve to direct the folding process towards the native state. Moreover, although the events in the folding of globular proteins appear to be diverse and complex [4], insights into their nature have begun to emerge from both experimental and theoretical studies, leading to optimism that the underlying principles of protein folding will soon become well established [5, 6]. These recent advances have led to the so-called 'new view' of protein folding [7, 8]. This new view, which is based on 'averaged effective energy surfaces' [3, 9] or 'energy landscapes' [8–10], replaces the concept of sequential events with that of parallel events, and describes the folding process as a progressive organization of an ensemble of partially folded structures. Individual molecules have individual trajectories on the effective energy surface, and although an ensemble of molecules explores a large number of conformations, any particular molecule samples only a very limited region of conformational space.

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The lysozyme story so far

Hen lysozyme is one of the best characterized and most studied of all biological macromolecules. This small monomeric protein of 129 residues (M_r 14305) contains many of the structural motifs commonly found in globular proteins (fig. 1). It is made up of two structural domains, one consisting of four α-helices and a C-terminal 3_{10} helix (referred to as the α-domain) and the other consisting of a triple-stranded antiparallel β-sheet, a 3_{10} helix and a long loop (the β-domain). Furthermore, a short double-stranded antiparallel β-sheet links these two domains, as does one of the four disulphide bridges. The kinetics of folding of this protein with its four disulphide bonds intact has been studied extensively using a variety of different techniques. Thus, by combining hydrogen-exchange labelling, using two-dimensional nuclear magnetic resonance (NMR) spectroscopy [13, 14] and electrospray ionization mass spectrometry (ESI-MS [15]), with stopped-flow absorbance, fluorescence, and near and far ultraviolet (UV) circular dichroism (CD) experiments [14, 16–20], it has been possible to propose a detailed model for the in vitro folding process of this enzyme [21, 22].

This folding process has been found to be a complex mechanism under most conditions. It appears not to be a simple sequential assembly process but to involve parallel events with distinct kinetic profiles. The earliest detected events [time constant (τ) < 2 ms] include both formation of extensive, although fluctuating, secondary structure (only marginal protection from hydrogen exchange is apparent at this stage [14, 23]), and a substantial degree of hydrophobic collapse. Far-UV CD experiments provide evidence for the formation of at least a substantial proportion of the native secondary structure within a few milliseconds after initiation of folding [14, 17–19], and various fluorescence parameters (intrinsic fluorescence intensity, anilinonaphthalenesulfonic acid (ANS) binding, and quenching of fluorescence by iodide ions) suggest that formation of a relatively condensed structure occurs on the same very short time scale [20]. Recent 'dead-time' labelling experiments [23] reveal the formation of an embryonic native-like structure in the α-domain at this very early stage of folding which, although only marginally stable, is probably significant in laying the foundations for the later stages of folding. Interestingly, such secondary structure with native-like character has been shown to speed up the search in the collapsed state for the native-like folding 'core' in simulations of the folding of a 125-mer protein [24]. The hydrophobically collapsed state which is formed at the onset of the folding process of lysozyme has been suggested to have properties characteristic of equilibrium molten globule states [14, 17–21, 23].

Following this initial collapse, kinetic heterogeneity is observed in the folding behaviour of lysozyme, and intermediate states become populated. About 15% of molecules refold in a very slow reaction with a time constant in the range of 20 s; folding of this population of molecules might be limited by *cis-trans* isomerization of one or both peptide bonds containing proline residues [16]. This very slow phase is not considered further in this paper. Hydrogen-exchange labelling studies [14, 15] indicate that at pH 5.5 and 20 °C the majority (70%) of the remaining molecules acquire extensive hydrogen-exchange protection in one of the two domains of the protein (the α-domain) before the other. For amides in this region, biphasic kinetics of protection are observed, with time constants of 5 to 10 ms and 65 ms, and average amplitudes of 20 and 50%, respectively. Thus, along this major folding route alternative 'subpathways' exist, both leading to the accumulation of a well-defined intermediate in which the α-domain is persistently structured in the absence of a stable β-domain. The amides located in the latter domain subsequently become protected from exchange in a slow and rate-limiting reaction ($\tau \approx 350$ ms). In this process, the two structural domains that make up the native fold (fig. 1) thus behave as distinct 'folding domains'. In addition, the similar rates of protection of different amides within both domains suggest that the formation of each domain is a highly cooperative process, which does not merely reflect the dif-

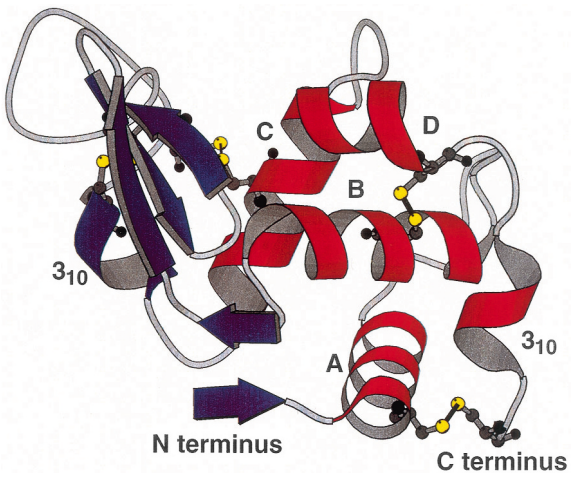

Figure 1. Schematic view of the structure of hen egg white lysozyme [11]. The two structural lobes in the native structure which form distinct folding domains are highlighted red (α-domain) and blue (β-domain). The four disulphide bonds are also shown. The diagram was drawn using the program MOLSCRIPT [12].

ference in the type of secondary structure in each domain [21].

The remaining 30% of molecules are observed to achieve a significant degree of protection against exchange in both domains on a much shorter time scale [15], forming apparently native-like structure with a time constant of $\sim$10 ms. Remarkably, this value corresponds to the folding rate observed in studies of a number of small single-domain proteins for which folding occurs by an effective two-state process without any detectable intermediates [22, 25, 26].

This kinetic partitioning between fast and slow refolding molecules appears to arise from conformational heterogeneity formed early in the folding process. Indeed, refolding experiments from denatured states having different conformational properties [27] indicate that these complexities in the folding arise from the ensemble of conformations resulting from the collapse process and not as a consequence of residual interactions in the denatured state. Similarly, recent stopped-flow, double-jump experiments [28] have confirmed that the kinetic complexity in lysozyme refolding is not due to a heterogeneous population of slowly interconverting unfolded species, for example arising from the occurrence of *cis* proline isomers in the unfolded state. Heterogeneity of the collapsed state and not of the unfolded state, therefore, provides a mechanism for the generation of kinetically distinct populations of folding molecules [22, 27], some of which already display substantial native-like features after the initial rapid collapse, while others need first to reorganize, a potentially slow reaction in a compact state [14]. The four preexisting disulphide bonds might be one of the determinants for the kinetic heterogeneity of the lysozyme folding process [19, 22].

The lysozyme story continued

In this paper, we summarize recent experiments which shed more light on the folding process of lysozyme, and we relate the findings from these experiments to the 'new view' of protein folding, and discuss the possible nature of the energy surface on which folding occurs.

Fast and slow tracks in lysozyme folding

One notable aspect of the lysozyme folding process is that on the fast folding track, along which about 30% of the molecules acquire native-like hydrogen-exchange protection in both the α- and β-domains with $\tau \approx 10$ ms, there is no detectable corresponding fast phase in near-UV CD experiments ([20]; A. Matagne, S. E. Radford and C. M. Dobson, unpublished data, fig. 2a). This results in uncertainty about the time scale of formation of the fully native functional enzyme. In an attempt to resolve this intriguing issue, we have investigated in detail the kinetics of binding of the competitive inhibitor MeU-diNAG (4-methylumbelliferyl-N,N′-diacetyl-β-D-chitobioside) during lysozyme folding [26]. In these experiments specific binding of this fluorescently labelled inhibitor to the enzyme active site, which lies between the two structural folding domains, has been used to determine directly the rate of formation of the functional native protein. The data at 20 °C (fig. 2b) indicate that two distinct kinetic events, with time constants and amplitudes of about 100 ms and 25% for the fast phase and 350 ms and 75% for the slow phase, can be detected in the MeU-diNAG binding kinetics. The striking correspondence in the amplitudes of the two phases judged by hydrogen exchange labelling and the inhibitor binding studies strongly suggests that both methods monitor the same populations of refolding molecules, which form the native state along two distinct folding tracks [26].

For those molecules folding along the fast track (ca. 25% of molecules), although hydrogen-exchange protection occurs for both domains in a fast phase ($\tau \approx 10$ ms), formation of the active site requires a subsequent step with $\tau \approx 100$ ms. We have proposed [26], therefore, that the fast track in refolding occurs via an intermediate which has extensive persistent native-like structure in both the α- and β-domains, but yet which is not entirely native since the active-site cleft is not formed as judged by its incapacity to bind the competitive inhibitor. This intermediate accumulates because it forms prior to the rate-limiting step, which may be associated with the reorganization and docking of the two partly struc-tured domains to form the active site and the fully native state. From these findings, it might be expected that multiexponential kinetics would also be observed when monitored by near-UV CD. However, the high level of complexity of the near-UV CD signal might well be the explanation for an apparently single exponential refolding process in the near-UV CD experiments [26].

Conversely, for those molecules folding along the slow track (ca. 75% of molecules), the coincidence between the rate of formation of the active site and that of the slow step of protection of amides in the β-domain ($\tau \approx 350$ ms) suggests that the active site could be achieved cooperatively with the formation of a native-like β-domain and its integration into the native structure. Alternatively, a subsequent step could occur sufficiently rapidly relative to the formation of the β-domain that it is not detected in the kinetic analysis. Interestingly, this would allow the $\tau \approx 100$ ms step observed on the fast track to be a feature of folding on the slow track. Further experiments are needed to distinguish between these two situations.

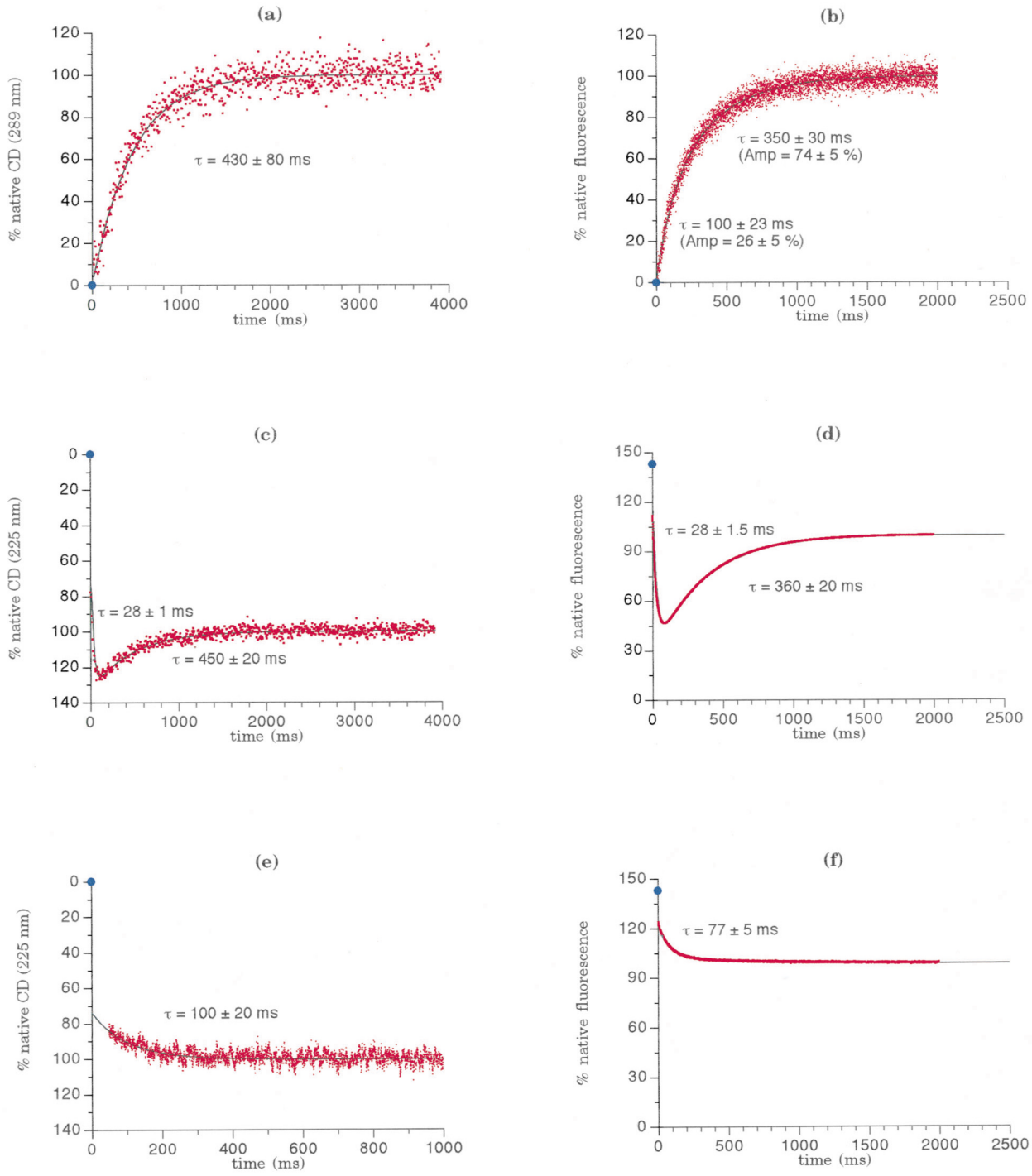

Figure 2. The folding kinetics of lysozyme initiated by 11-fold dilution (pH 5.5) from 6 M GndHCl at 20 °C (*a*–*d*) and 50 °C (*e*–*f*). Time constants and amplitudes are given here with errors representing standard deviations. Data have been normalized such that the value of the native state is 100%; in each case, the value of the denatured state is shown (●). (*a*) Near-UV CD (289 nm); (*b*) binding of the fluorescent inhibitor MeU-diNAG; (*c*, *e*) far-UV CD (225 nm); (*d*, *f*) intrinsic tryptophan fluorescence (adapted from [26, 31]).

Recently, a new molecular dynamics procedure [29] has allowed an investigation of the hen lysozyme unfolding process. This approach, which is based on the artificial insertion of water molecules into suitable cavities arising in the protein structure during unfolding, indicates that the first – rate-limiting – transition corresponds to

the 'unlocking' of the two lysozyme folding domains. The resulting compact 'unlocked' structure maintains much of the native secondary structure but loses many fixed tertiary interactions and the integrity of the enzyme active site, and thus appears to be similar to that suggested for the late folding intermediate occurring on the fast, and possibly on the slow, folding tracks [26, 29].

Non-native interactions and cooperativity in the lysozyme folding process

Stopped-flow optical experiments have played an important role in understanding the folding pathway of lysozyme [14, 17, 19, 20]. The refolding kinetics of lysozyme at 20 °C monitored by intrinsic fluorescence and far-UV CD are illustrated in figure 2c, d. With both techniques a significant dead-time event is observed, which corresponds to the formation of the collapsed state described above. Following this very rapid event, development of excess negative ellipticity occurs in the far-UV CD, whereas quenching of the tryptophan fluorescence results in a minimum in the intrinsic fluorescence intensity. In both cases these phases ($\tau \approx 30$ ms) reach a maximum amplitude at ~ 100 ms. In the third and slowest phase ($\tau \approx 400$ ms) each signal attains its final native-state value. Remarkably, the formation of significant hydrogen-exchange protection in the α-domain of lysozyme during folding occurs on a time scale similar to that of the development of excess negative ellipticity in the far-UV CD and of the quenching of tryptophan fluorescence intensity to an extent greater than that of the native state. The similarity in the kinetics suggests that both techniques are monitoring the same series of events during the folding process; and even though there has been no definitive evidence for the molecular origin of these events, these steps have commonly been attributed to the formation of the α-domain intermediate and the subsequent formation of the native state (see e.g. refs 20, 25, 30).

Recently, by changing the refolding conditions, we have demonstrated that the accumulation of the α-domain intermediate and the development of both the overshoot in the far-UV CD and the minimum in the fluorescence intensity are indeed highly correlated [31]. Stopped-flow fluorescence and CD spectroscopy were used in conjunction with quenched-flow hydrogen-exchange labelling, monitored by ESI-MS, to compare the refolding kinetics at 20 and 50 °C. At 50 °C both the overshoot in the far-UV CD and the minimum in fluorescence intensity disappear, and the major phase observed at 50 °C in both experiments fits to a single exponential process with a time constant of 80 to 100 ms (fig. 2e, f). In addition, hydrogen-exchange labelling experiments monitored by ESI-MS carried out at the two temperatures show significant differences (fig. 3). At 20 °C (fig. 3a) three well-defined species with different masses can be detected over the time course of the experiment [15]. One of them corresponds to the unprotected enzyme (i.e. the fully unfolded state) and is denoted P0. Another peak, P50, which is 50 Da higher in mass, corresponds to the fully protected state (i.e. the native-like state) which protects 50 sites significantly from exchange. The third peak, P28, which shows intermediate protection between the unprotected and the fully protected states, corresponds to the α-domain intermediate (28 protected amides) which accumulates transiently along the slow folding track.

At 50 °C (fig. 3b) there is clear evidence for distinct fast and slow refolding populations, as observed at 20 °C, although folding occurs significantly more rapidly. The folding process is, however, substantially more cooperative at 50 °C, and in particular, the α-domain intermediate is not detected by hydrogen-exchange labelling at 50 °C. Instead, the kinetics of the disappearance of P0 correlate closely with the appearance of P50 ($\tau \approx 6$ and ≈ 140 ms for the fast and slow phases, respectively), whereas at 20 °C the unprotected state (P0) decreases in intensity in two phases ($\tau \approx 10$ and ≈ 100 ms) much faster than the fully protected state (P50) appears ($\tau \approx 16$ and ≈ 350 ms). Addition of 2 M NaCl, however, restores at 50 °C the characteristic minimum in fluorescence intensity observed at 20 °C and the protection associated with the α-domain intermediate (fig. 3c). The presence of a high salt concentration appears to stabilize the intermediate significantly. That fluorescence and hydrogen-exchange protection change concomitantly therefore strongly suggests that the accumulation of the α-domain intermediate and the development of unusual optical properties observed during refolding are highly correlated [31].

These results support the view that this transient species contains non-native interactions [14, 19, 32, 33] and indicate that the rate-determining step in the formation of the native state for the slowly folding population of molecules involves structural reorganization. This is likely to be associated with the formation and stabilization of structure within the β-domain, and from rearrangement of the interfacial region between the two domains. Therefore, the α-domain intermediate is not seen as a kinetically trapped intermediate which slows the refolding process [28]. Rather, it accumulates simply because of its intrinsic stability even in the absence of the fully folded β-domain, for which the formation of structure is rate limiting. In this respect, it is remarkable that at 50 °C, where the α-domain is not detected, we

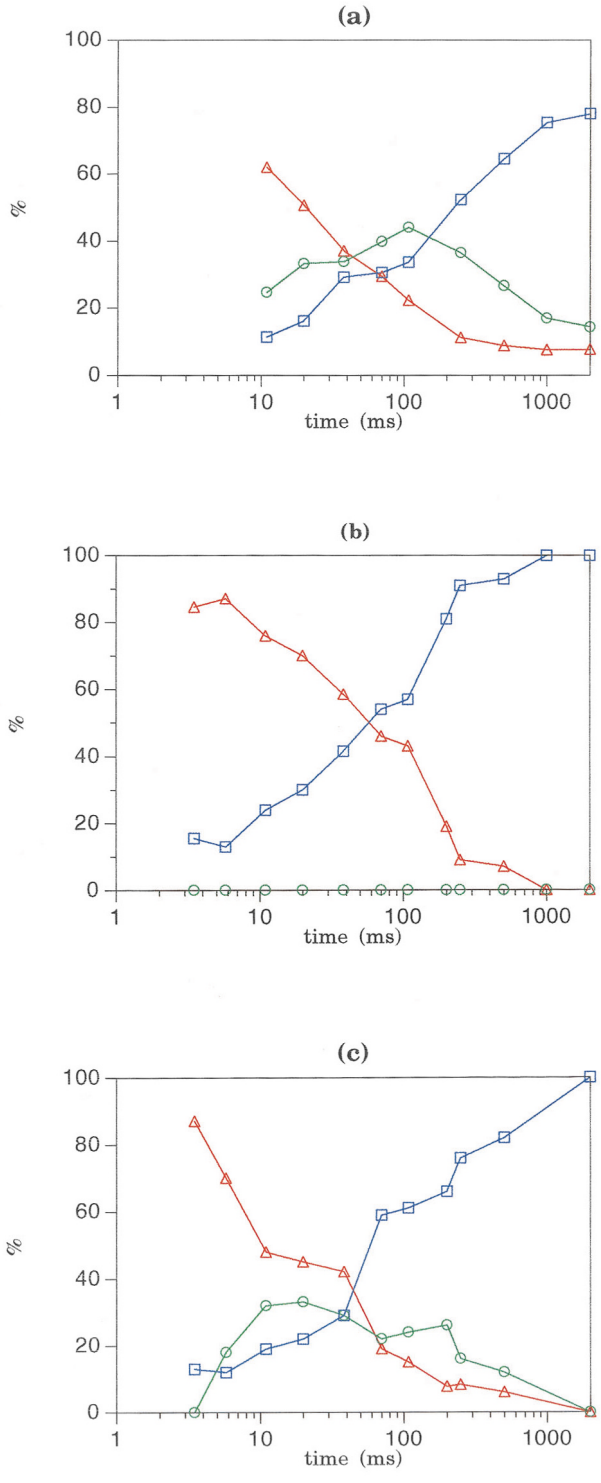

Figure 3. Refolding kinetics of lysozyme at 20 °C (*a*) and 50 °C in the absence (*b*) and in the presence (*c*) of 2 M NaCl as monitored by hydrogen-exchange labelling and ESI-MS. Time courses of the population of the unprotected state (△, P0), the α-domain intermediate (○, P28) and species with native-like protection (□, P50) are shown (adapted from [31]).

observe a kinetic heterogeneity which is reminiscent of that observed at 20 °C, where the intermediate is highly populated. These findings indicate that destabilization of the α-domain intermediate does not suppress the slow folding track, and thus support the contention that this folding intermediate does not by itself generate a kinetic trap to slow the folding reaction [31].

Schematic energy surface for lysozyme folding

The folding of proteins is increasingly being viewed in the context of energy surfaces or landscapes [6, 8,10, 34]. In this view of folding, intermediates result from the existence of barriers that generate local minima in the energy surface. There is no need for the concept of on- or off-pathway species; fast and slow folding populations can simply result from the distributions of molecules encountering such minima [8, 35]. In the case of lysozyme under the conditions of the present experiments, folding of the majority of molecules at both 20 and 50 °C appears to be limited by the slow folding of the region of the polypeptide chain that corresponds to the β-domain. Whether this is associated with the need to reorganize alternative global folds, or more local features such as alternative conformations of side chains or of disulphide bonds, is not yet clear. But the experiments discussed here suggest that formation of stable native-like structure in the α-domain does not by itself result in barriers to rearrangement that limit the folding reaction.

Recently, a schematic energy surface (fig. 4) has been proposed for lysozyme [6] which is broadly consistent with the experimental data for lysozyme discussed in this article. The general format of this surface is based on the results of simulations using simple lattice models to describe folding, and represents the averaged effective energy (E) of folding as a function of a progress variable, Q, which is the fraction of contacts present in the native structure that are formed at a given stage of folding. The third axis, P, is related to the configurational entropy of the system and describes the extent of conformational space accessible to the polypeptide chain [6]. This schematic surface (fig. 4) is designed to illustrate at least in outline the three major experimental features of the lysozyme folding reaction (at 20 °C), i.e. the early collapse with very rapid formation of native-like secondary structure, the sampling of distinct intermediate species during folding and the existence of complex kinetics arising from partitioning between fast and slow populations of refolding molecules. Thus, significant minima can be seen along the effective energy surface prior to the transition region for the formation of the native state, and there are fast and slow

trajectories separated by a 'ridge' which inhibits exchange between them.

The early collapse of the polypeptide chain is represented by the steep drop at the top of figure 4. That this is indicated to occur at relatively low values of Q reflects the fact that few native-like contacts may be needed for collapse to occur; the driving force is likely to be simply the burial of hydrophobic groups [6, 36]. From this highly heterogeneous collapsed state, nativelike structure develops independently in the two structural domains, and hence intermediates are formed. Along the slow folding track, the faster folding of the α-domain relative to the β-domain results in a partially folded state with native-like structure within one of the two domains of the overall fold. In the energy surface of figure 4 this corresponds to the deep minimum on the right-hand side. Conversely, along the fast folding track, the intermediate state with extensive native-like structure within both domains is represented by the minimum on the left-hand side of the diagram. A barrier is represented in this region to reflect the fact that the two partly structured domains coalesce in a slower step which results in the formation of the fully functional native state.

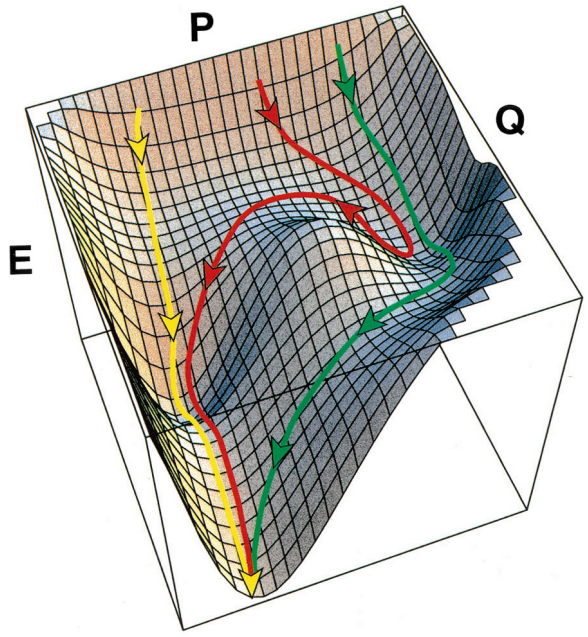

Figure 4. Schematic averaged effective energy surface based on experimental data for lysozyme folding. The averaged effective energy (E) and a parameter P related to the configurational entropy of the system are shown as a function of the progress variable Q, which corresponds to the fraction of native contacts formed in the refolding molecules. Several possible folding trajectories are show: a fast folding trajectory (yellow); a slow folding trajectory that crosses the high energy barrier (green); a slow folding trajectory that returns to a less folded state and then follows the valley corresponding to the fast folding trajectory (red). Reprinted with permission from: Dobson C. M., Sali A. and Karplus M. (1998) Protein folding: a perspective from theory and experiment. Angew. Chem. **100:** 4000–4025, © 1998 Academic Press, London.

Experimental findings imply that the rate-limiting step in the folding process for the slow folding population of lysozyme molecules involves the reorganization of misfolded species [14, 31, 33]. Such rearrangement processes are likely to be subject to significant barriers associated with interactions within the collapsed polypeptide chain [6]. Thus, the slow folding species have to go over a higher barrier to reach the native state (fig. 4, green trajectory) or to unfold and return to the fast folding pathway (fig. 4, red trajectory). The details of such events are not clear, although it is apparent from the experimental hydrogen-exchange data that for most molecules significant unfolding of the α-domain itself does not occur once it has formed [31]. The schematic surface shown in figure 4 reconciles at least qualitatively both the kinetic partitioning and the distinct intermediates characterizing the lysozyme folding process. It emphasizes the formation at the onset of the reaction of a largely heterogeneous collapsed state with few specific interactions. It is at this stage that kinetic partitioning occurs with the subsequent population of intermediate states. That parallel folding routes exist is due to significant barriers to the interconversion between slow and fast folding populations.

This surface is, however, constructed to explain the folding behaviour of lysozyme under a given set of conditions. If these are altered, the surface will be altered, and changes in the folding behaviour will occur. For example, at 20 °C the minima on the surface are sufficiently deep that intermediates can accumulate. At 50 °C, however, experiments show that the α-domain intermediate does not accumulate except in the presence of high concentrations of salt, conditions known to stabilize other partially folded states of proteins [37]. Presumably in the absence of salt, the interactions stabilizing this intermediate are not sufficient to offset the increased configuration entropy of a polypeptide chain at higher temperature. Other features of the surface, for example that enabling kinetic partitioning to occur early in folding, appear not to be changed significantly by the increase in temperature from 20 to 50 °C. Variation in solution conditions, along with other approaches such as site-directed mutagenesis, will undoubtedly enable the energy surfaces, and the interactions that they reflect, to be probed in greater detail.

Conclusions

The energy surface discussed here is highly schematic and designed to begin to provide a framework on which

experimental and theoretical ideas can develop. The 'new view' of folding does not, as figure 4 indicates, replace the ideas of pathways and intermediates, but places them within the context of an ensemble picture of the conformations present during a folding reaction. In this article, we have tried to indicate how the complexities in the experimental data for lysozyme can be rationalized at least in outline in terms of a relatively simple energy surface. The challenge now is to find methods of providing much greater detail about the events occurring in folding, and to relate these to the specific characteristics of the protein sequence. In particular, we need to develop structured approaches to allow folding to be probed at the level of individual residues. NMR spectroscopy represents a particularly promising approach for progress towards this objective [38], along with strategies based on protein-engineering methodologies [39].

As part of these general approaches to mapping the surface of folding, the study of intermediates trapped by mechanisms such as those described in this paper is important, as it can provide considerable insight into the nature of incompletely folded species which under other circumstances are too transient to characterize in any detail. In the case of lysozyme, for example, the ability to study the α-domain intermediate reveals that formation of the different characteristics of the native structure can be decoupled. In particular, it is clear that native-like secondary structure and an overall native fold, revealed by highly protected amide hydrogens, can form during folding in the absence of the close packing and high cooperativity that are characterized by the near-UV CD and fluorescence properties of aromatic residues. Such intermediate species, which have been observed for a number of proteins [40, 41], have become of particular interest in the case of lysozyme, not just because of the insight that they give into the nature of the folding process, but also because their stabilization in variant forms of the human protein resulting from single point mutations has recently been implicated in the formation of fibrils associated with familial amyloidosis [42]. Further study of the folding of model systems such as lysozyme, therefore, promises to have significance beyond that associated with the unravelling of the fundamental principles of folding.

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1 Anfinsen C. B. (1973) Principles that govern the folding of protein chains. Science **181:** 223–230
2 Levinthal C. (1968) Are there pathways for protein folding? J. Chim. Phys. **65:** 44–45
3 Sali A., Shakhnovich E. and Karplus M. (1994) How does a protein fold? Nature **369:** 248–251
4 Ptitsyn O. B. (1996) A determinable but unresolved problem. FASEB J. **10:** 3–4
5 Fersht A. R. (1997) Nucleation mechanisms in protein folding. Curr. Opin. Struct. Biol. **7:** 3–9
6 Dobson C. M., Sali A. and Karplus M. (1998) Protein folding: a perspective from theory and experiment. Angew. Chem. **100:** 4000–4025
7 Baldwin R. L. (1995) The nature of protein folding pathways: the classical view versus the new view. J. Biomol. NMR **5:** 103–109
8 Dill A. K. and Chan H. S. (1997) From Levinthal to pathways to funnels. Nature Struct. Biol. **4:** 10–19
9 Bryngelson J. D., Onuchic J. N., Socci N. D. and Wolynes P. G. (1995) Funnels, pathways and the energy landscape of protein folding: a synthesis. Prot. Struct. Funct. Gen. **21:** 167–195
10 Wolynes P. G., Luthey-Schulten Z. and Onuchic J. N. (1996) Fast-folding experiments and the topography of protein folding energy landscapes. Chem. Biol. **3:** 425–432
11 Blake C. C. F., Koenig D. F., Mair G. A. and Sarma R. (1965) Crystal structure of lysozyme by X-ray diffraction. Nature **206:** 757–761
12 Kraulis P. J. (1991) Molscript: a program to produce both detailed and schematic plots of protein structures. J. Appl. Cryst. **24:** 946–950
13 Miranker A., Radford S. E., Karplus M. and Dobson C. M. (1991) Demonstration by NMR of folding domains in lysozyme. Nature **349:** 633–636
14 Radford S. E., Dobson C. M. and Evans P. A. (1992) The folding pathway of hen lysozyme involves partially structured intermediates and multiple pathways. Nature **358:** 302–307
15 Miranker A., Robinson C. V., Radford S. E., Aplin R. T. and Dobson C. M. (1993) Detection of transient protein folding populations by mass spectrometry. Science **262:** 896–900
16 Kato S., Shimamoto N. and Utiyama H. (1982) Identification and characterization of the direct folding process of hen egg white lysozyme. Biochemistry **21:** 38–43
17 Kuwajima K., Hiraoka Y., Ikeguchi M. and Sugai S. (1985) Comparison of the transient folding intermediates in lysozyme and α-lactalbumin. Biochemistry **24:** 874–881
18 Ikeguchi M., Kuwajima K., Mitani M. and Sugai S. (1986) Evidence for identity between the equilibrium unfolding intermediate and a transient folding intermediate: a comparative study of the folding reactions of α-lactalbumin and lysozyme. Biochemistry **25:** 6965–6972
19 Chaffotte A. F., Guillou Y. and Goldberg M. E. (1992) Kinetic resolution of peptide bond and side chain far UV circular dichroism during the folding of hen egg white lysozyme. Biochemistry **31:** 9694–9702
20 Itzhaki L. S., Evans P. A., Dobson C. M. and Radford S. E. (1994) Tertiary interactions in the folding pathway of hen lysozyme: kinetic studies using fluorescent probes. Biochemistry **33:** 5212–5220
21 Dobson C. M., Evans P. A. and Radford S. E. (1994) Understanding how proteins fold: the lysozyme story so far. Trends Biochem. Sci. **19:** 31–37
22 Radford S. E. and Dobson C. M. (1995) Insights into protein folding using physical techniques: studies of lysozyme and α-lactalbumin. Phil. Trans. R. Soc. Lond. **B348:** 17–25

23 Gladwin S. T. and Evans P. A. (1996) Structure of very early protein folding intermediates: new insights through a variant of hydrogen exchange labelling. Fold. Des. **1:** 407–417
24 Dinner A. R., Sali A. and Karplus M. (1996) The folding mechanism of larger model proteins: role of native structure. Proc. Natl. Acad. Sci. USA **93:** 8356–8361
25 Kiefhaber T. (1995) Kinetic traps in lysozyme folding. Proc. Natl. Acad. Sci. USA **97:** 9029–9033
26 Matagne A., Radford S. E. and Dobson C. M. (1997) Fast and slow tracks in lysozyme folding: insight into the role of domains in the folding process. J. Mol. Biol. **267:** 1068–1074
27 Kotik M., Radford S. E. and Dobson C. M. (1995) Comparison of the refolding of hen lysozyme from dimethyl sulfoxyde and guanidinium chloride. Biochemistry **34:** 1714–1724
28 Wildegger G. and Kiefhaber T. (1997) Three-state model for lysozyme folding: triangular folding mechanism with energetically trapped intermediate. J. Mol. Biol. **270:** 294–304
29 Williams M. A., Thornton J. M. and Goodfellow J. M. (1997) Modelling protein unfolding: hen egg white lysozyme. Prot. Eng. **10:** 895–903
30 Parker M. J., Spencer J. and Clarke A. R. (1995) An integrated kinetic analysis of intermediates and transition states in protein folding reactions. J. Mol. Biol. **253:** 771–786
31 Matagne A., Chung E. W., Ball L. J., Radford S. E., Robinson C. V. and Dobson C. M. (1998) The origin of the α-domain intermediate in the folding of hen lysozyme. J. Mol. Biol. **277:** 997–1005
32 Denton M. E., Rothwarf D. M. and Scheraga H. A. (1994) Kinetics of folding of guanidine-denatured hen egg white lysozyme and carboxymethyl (Cys^{6},Cys^{127})-lysozyme: a stopped flow absorbance and fluorescence study. Biochemistry **33:** 11225–11236
33 Rothwarf D. M. and Scheraga H. A. (1996) Role of non-native aromatic and hydrophobic interactions in the folding of hen egg white lysozyme. Biochemistry **35:** 13797–13807
34 Karplus M. and Sali A. (1995) Theoretical studies of protein folding and unfolding. Curr. Opin. Struct. Biol. **5:** 58–73
35 Guo Z. and Thirumalai D. (1995) Kinetics of protein folding: nucleation mechanism, time scales and pathways. Biopolymers **36:** 83–102
36 Dill K. A., Fiebig K. M. and Chan H. S. (1993) Cooperativity in protein-folding kinetics. Proc. Natl. Acad. Sci. USA **90:** 1942–1946
37 Fink A. L. (1995) Compact intermediate states in protein folding. Annu. Rev. Biophys. Biomol. Struct. **24:** 495–522
38 van Nuland N. A. J., Forge V., Balbach J. and Dobson C. M. (1998) Real-time NMR studies of protein folding. Acc. Chem. Res., in press
39 Fersht A. R. (1995) Characterizing transition states in protein folding: an essential step in the puzzle. Curr. Opin. Struct. Biol. **5:** 79–84
40 Baldwin R. L. (1993) Pulsed H/D-exchange studies of folding intermediates. Curr. Opin. Struct. Biol. **3:** 84–91
41 Roder H. and Elöve G. A. (1994) Early stages of protein folding. In: Mechanisms of Protein Folding, Pain R. H. (ed.), pp. 26–54, Oxford University Press, Oxford
42 Booth D. R., Sunde M., Bellotti V., Robinson C. V., Hutchinson W. L., Fraser P. E. et al. (1997) Instability, unfolding and aggregation of human lysozyme variants underlying amyloid fibrilogenesis. Nature **385:** 787–793

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