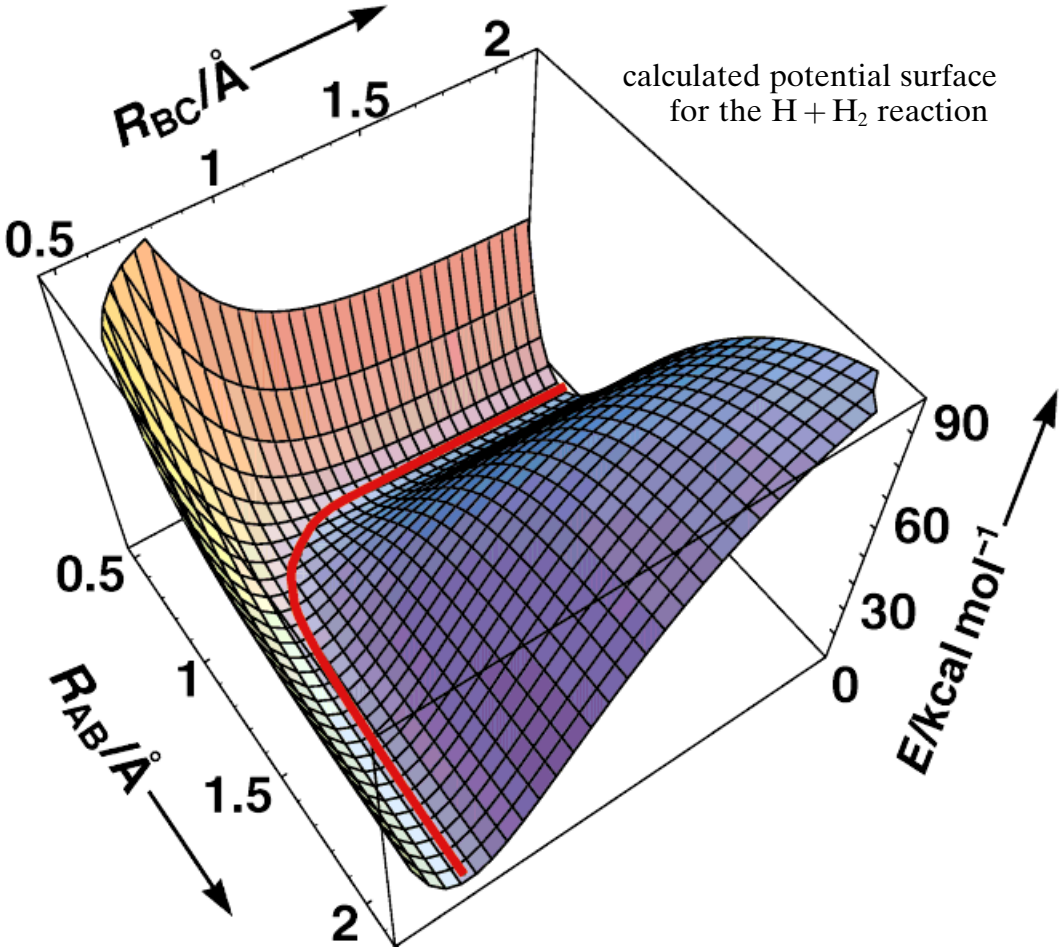
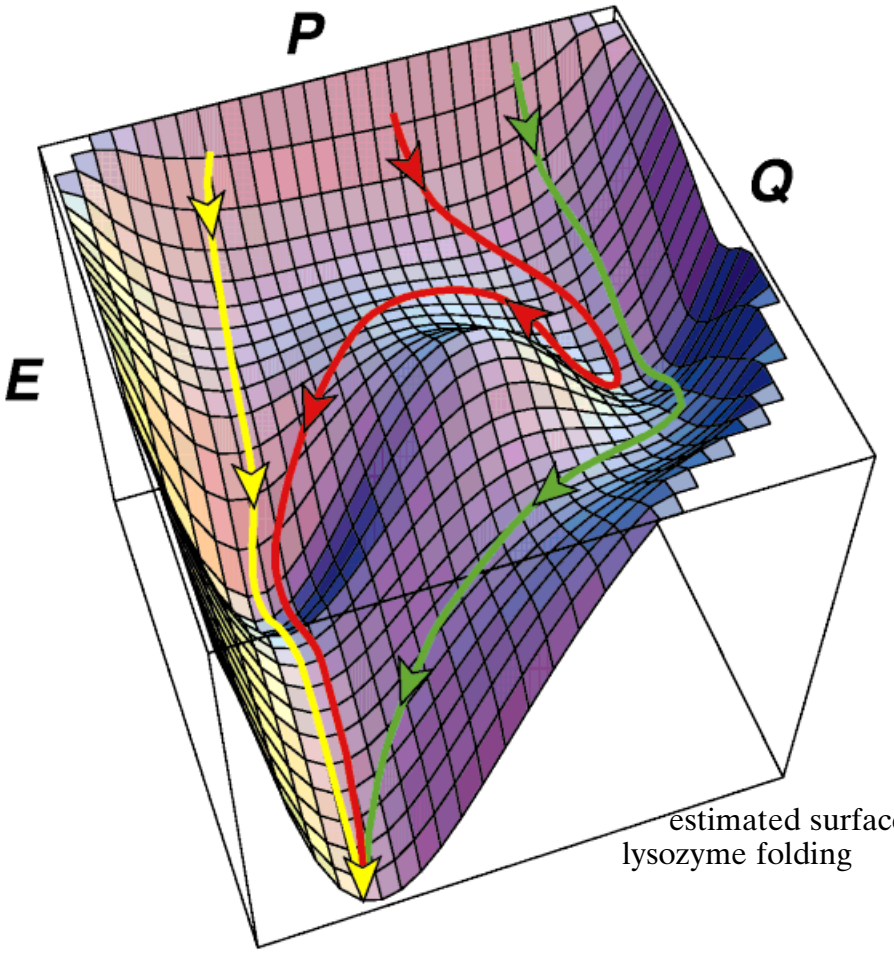


calculated potential surface for the $H + H_2$ reaction

A knowledge of the potential energy surface that determines the dynamics of a polypeptide chain is as important for understanding protein folding as is the potential surface for the $H + H_2$ reaction.



estimated surface for lysozyme folding

Protein Folding: A Perspective from Theory and Experiment

Christopher M. Dobson,* Andrej Šali, and Martin Karplus*

Proteins are involved in virtually every biological process, and their functions range from catalysis of chemical reactions to maintenance of the electrochemical potential across cell membranes. They are synthesized on ribosomes as linear chains of amino acids in a specific order from information encoded within the cellular DNA. To function, it is necessary for these chains to fold into the unique native three-dimensional structure that is characteristic for each protein. This involves a complex molecular recognition phenomenon that depends on the cooperative action of many relatively weak nonbonding interactions. As the number of possible conformations for a polypeptide chain is astronomically large, a systematic search for the native (lowest energy) structure would require an almost infinite length of time. Recently, significant progress has been made towards solving this paradox and understanding the mechanism of folding. This has come about through advances in experimental strategies for following the folding reactions of proteins in the laboratory with biophysical techniques, and through progress in theoretical approaches that simulate the folding process with simplified models. The most recent advances in this area are comparable in significance to those that took place in developing an understanding of reactions of small molecules some thirty years ago. In this article we review the present state of our knowledge of the protein folding reaction and compare the concepts that are emerging with those that are now established for simpler reactions. A major distinction between protein folding and reactions of small molecules is the heterogeneity of the folding process and the resulting complex interplay between entropic and enthalpic contributions to the free energy of the system during the reaction. A unified model for protein folding is outlined based on the effective energy surface of a polypeptide chain and its bias towards the native state. An understanding of folding is important for the analysis of many events involved in cellular regulation, the design of proteins with novel functions, the utilization of sequence information from the various genome projects, and the development of novel therapeutic strategies for treating or preventing debilitating human diseases that are associated with the failure of proteins to fold correctly.

Keywords: kinetics · molecular dynamics · protein folding · proteins · structure elucidation

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1. Introduction

Reactions are central to chemistry. They range from simple exchange processes involving only a few atoms to protein folding, in which thousands of atoms take part. The understanding of a reaction is based on a knowledge of the role of the dominant interactions that determine the potential energy surface and a description of the dynamics leading from the reactants to the products. Substantial progress has been made in the past thirty years toward achieving this understanding for simple reactions through a combination of experimental developments and theoretical advances.[1] By contrast, protein folding is so complex that even the approaches to be used for its characterization are not fully defined.[2, 3] In this review, we begin by addressing some of the similarities and differences between simple chemical reactions and protein folding. Apart from the much greater complexity of protein folding, which requires the introduction of simplified theoretical models and

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novel experimental approaches, a number of more general issues arise. Not the least of these is the presence of an ensemble of molecules differing substantially in their structure and dynamics at various stages of the folding reaction.

Globular proteins are synthesized as linear chains of amino acids. To carry out their functions, they must fold rapidly and reliably to a specific structure designed by evolution for the particular task (Figure 1). Although the folding process in a cell involves a range of catalytic and control systems,[4] the information for folding is contained in the amino acid sequence for many, if not all, proteins.[5] A protein sequence must satisfy two requirements: one thermodynamic and one kinetic.[2, 6] The thermodynamic requirement is that the molecules adopt a unique folded conformation (the native state) which is stable under physiological conditions. The kinetic requirement is that the denatured polypeptide chain can fold into the native conformation within a reasonable time. A polypeptide chain can adopt so many conformations that there must be a way to reach the native state in a time many orders of magnitude shorter than that required for a random search.[7] To obtain an estimate of this order of magnitude, one can assume that there are three configurations for each amino acid residue. If a protein is made up of a polypeptide chain consisting of 100 amino acids, there is a total of about 10^{49} conformations. If only 10^{-11} s were required to convert from one conformation into another, a random search of all conformations would still require 10^{36} s or about 10^{29} years. This led Levinthal to suggest "pathways" for protein folding.[8] Many theoretical attempts to "fold" pro-

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Christopher Martin Dobson, born in Rinteln (Germany) in 1949, studied chemistry at the University of Oxford and completed his D. Phil. thesis on the NMR spectroscopy of proteins under the supervision of Professor R. J. P. Williams in 1975. After research fellowships in Oxford he moved to Harvard University in 1977 as an Assistant Professor of Chemistry. In 1980 he returned to the University of Oxford as a Lecturer and then Reader in Chemistry. He is presently Professor of Chemistry and Director-elect of the Oxford Centre for Molecular Sciences. In 1992 he was appointed a Howard Hughes International Research scholar, and in 1996 he was elected a Fellow of the Royal Society. His research interests have been focused on the development and application of NMR and other biophysical methods to biological and chemical problems. His recent activities have been directed particularly at unravelling the underlying mechanism of protein folding and determining its relevance to biological function and disease.

Andrej Šali, born in 1963 in Kranj (Slovenia), studied chemistry at the University of Ljubljana. He obtained his Ph.D. in 1991 at Birkbeck College, University of London with Professor T. L. Blundell in the area of comparative protein structure modelling. During a postdoctoral fellowship with Professor M. Karplus at Harvard University, he worked in the area of protein folding simulations. Since 1995 he has been an Assistant Professor at The Rockefeller University and is focusing on developing comparative modelling of protein structures and its applications in genomic research.

Martin Karplus, born in Vienna (Austria) in 1930, received his B.A. from Harvard College in 1950 and his Ph.D. from the California Institute of Technology in 1953. He worked at Oxford University as an National Science Foundation postdoctoral fellow from 1953 until 1955, when he joined the faculty of the University of Illinois. In 1960 Karplus became a professor at Columbia University, and in 1966 a professor at Harvard University. He was named Theodore William Richards Professor of Chemistry there in 1979. He is also Professeur Conventionné at the Université Louis Pasteur in Strasbourg. He is a member of the National Academy of Sciences (USA), the American Academy of Arts and Sciences, and the Netherlands Academy of Arts and Sciences. Early in his career Karplus studied magnetic resonance spectroscopy; of particular interest was his theoretical analysis of nuclear spin – spin coupling constants. He made fundamental contributions to the theory of reactive collisions between small molecules based upon trajectory calculations. He was one of the first researchers to apply many-body perturbation theory to atomic and molecular systems. He has studied reactions in biological molecules, such as the bonding of oxygen to hemoglobin and the photochemistry of visual pigments. His more recent work has been concerned with the development of molecular dynamics and other simulation methods for the elucidation of the properties of proteins and nucleic acids. He is now studying enzymatic reactions, protein folding, and the application of computational methods to combinatorial ligand design.

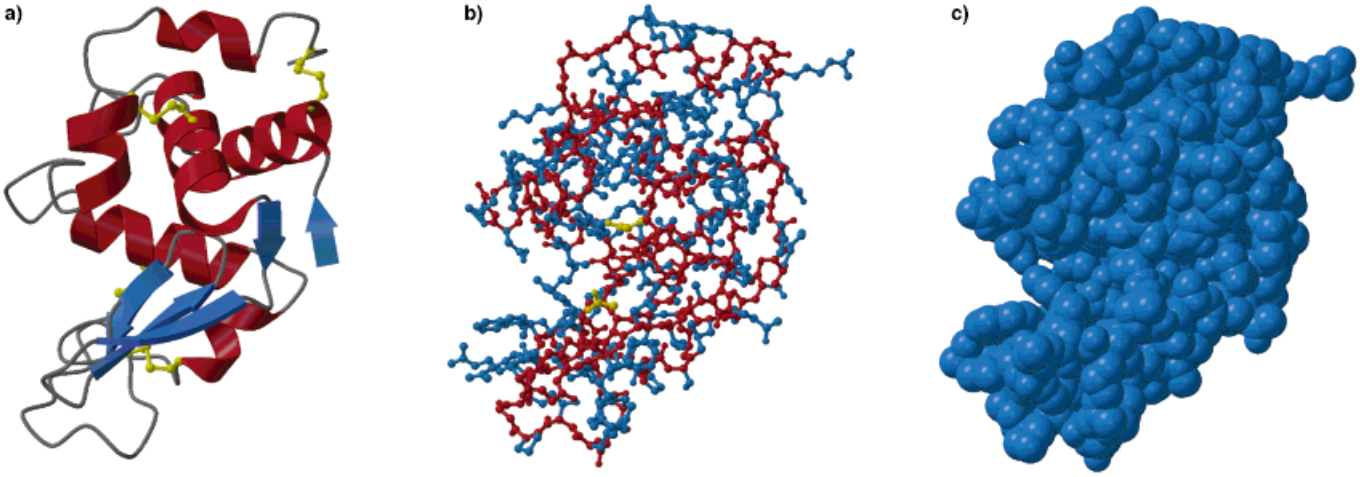


Figure 1. Different representations of the protein lysozyme (PDB code: 8LZT) drawn with the programs Molscript[163] and Render.[164] a) Ribbon diagram of the fold of the main chain of the protein with 129 residues. The α helices are shown as red spirals, and the β strands as blue arrows. The cross-links due to the disulfide bonds are shown in yellow. b) Ball-and-stick drawing in which the main chain is red, the side chains are blue, and the side chains involved in the catalytic mechanism are yellow. c) Space-filling view showing that the protein is a close-packed structure. Calculations with normal van der Waals radii indicate that more than 75 % of space is occupied.

teins based on the pathway concept have been made, but no one has succeeded in doing so,[9] in spite of some claims to the contrary. The difference between the limitations of what can be done on a computer in thousands of hours and what nature can do in less than a second clearly poses the question of the mechanism of protein folding, a question that has perplexed researchers for many decades.[10, 11]

Despite the long-standing interest in protein folding, it is only relatively recently that experiments to probe the details of the events that occur during the folding process have begun to be carried out.[3] As was the situation for reactions of small molecules thirty years ago,[12, 13] protein folding is now at the stage where theory and experiment can together make rapid progress toward an understanding of this complex process. Toward this end, a framework must be developed for interpreting the results of the new experimental techniques and for probing specific features of the folding reaction. In this review, we briefly outline some aspects of the reactions of small molecules to permit us to contrast them with the protein folding reaction. We then show how folding can be described in terms of existing theoretical and experimental results. Although the differences among proteins are often emphasized, the characteristics that emerge point to an underlying universality of the mechanism of folding when it is interpreted in terms of an averaged effective energy and the associated configurational entropy.

Because of recent theoretical and experimental advances in methods for studying the mechanism of protein folding, a review of this rapidly developing field addressed to chemists is particularly timely. Readers may wish to refer also to a number of other reviews directed more toward biologists and biophysicists that have appeared recently;[6, 14–17] they can serve to complement the present article. The viewpoint presented emphasizes our own work and experience, although we have profited greatly from the contributions of many others, cited in the list of references, to this exciting and rapidly developing field.

2. Energy Surfaces for Reactions of Small Molecules

The nature of any reaction, whether of a small molecule or a protein, is determined by the potential energy surface and the laws of dynamics.[1, 12, 13] The detailed experimental and theoretical analysis of simple chemical reactions is based on well-defined procedures. We briefly review them here to contrast the situation with that for proteins. Experimentally, the potential energy surface for a reaction is usually characterized first in a relatively crude way by measuring the reaction rate as a function of temperature to evaluate the activation energy E_A and the pre-exponential factor $A(T)$ in the Arrhenius formula; that is, the temperature-dependent rate constant is written as Equation (1). Most of the temper-

$$k(T) = A(T)\exp(-E_A/RT) \quad (1)$$

ature dependence arises from the exponential (Arrhenius) factor E_A/RT, whereas the factor $A(T)$ generally has only a weak temperature dependence. Measurements of $A(T)$ and E_A can be complemented by reactive scattering data obtained from studies with crossed molecular beams[12] and by measurements that permit direct observation of the transition state.[18] Such studies provide the details necessary to characterize the potential energy surface. Theoretical calculations can also be used to determine the potential energy surface, the reaction coordinate, and the transition state.[19] Finally, trajectory calculations, or their quantum-dynamical analogues, can be used to determine the overall rate constant and to simulate more detailed reactive scattering behavior.[13, 20] For a small number of simple reactions, the available theoretical descriptions are as accurate as the experimental results.[1, 20]

We consider briefly the simplest and best understood chemical reaction, the bimolecular exchange of a hydrogen atom with a hydrogen molecule. Only three internal coor-

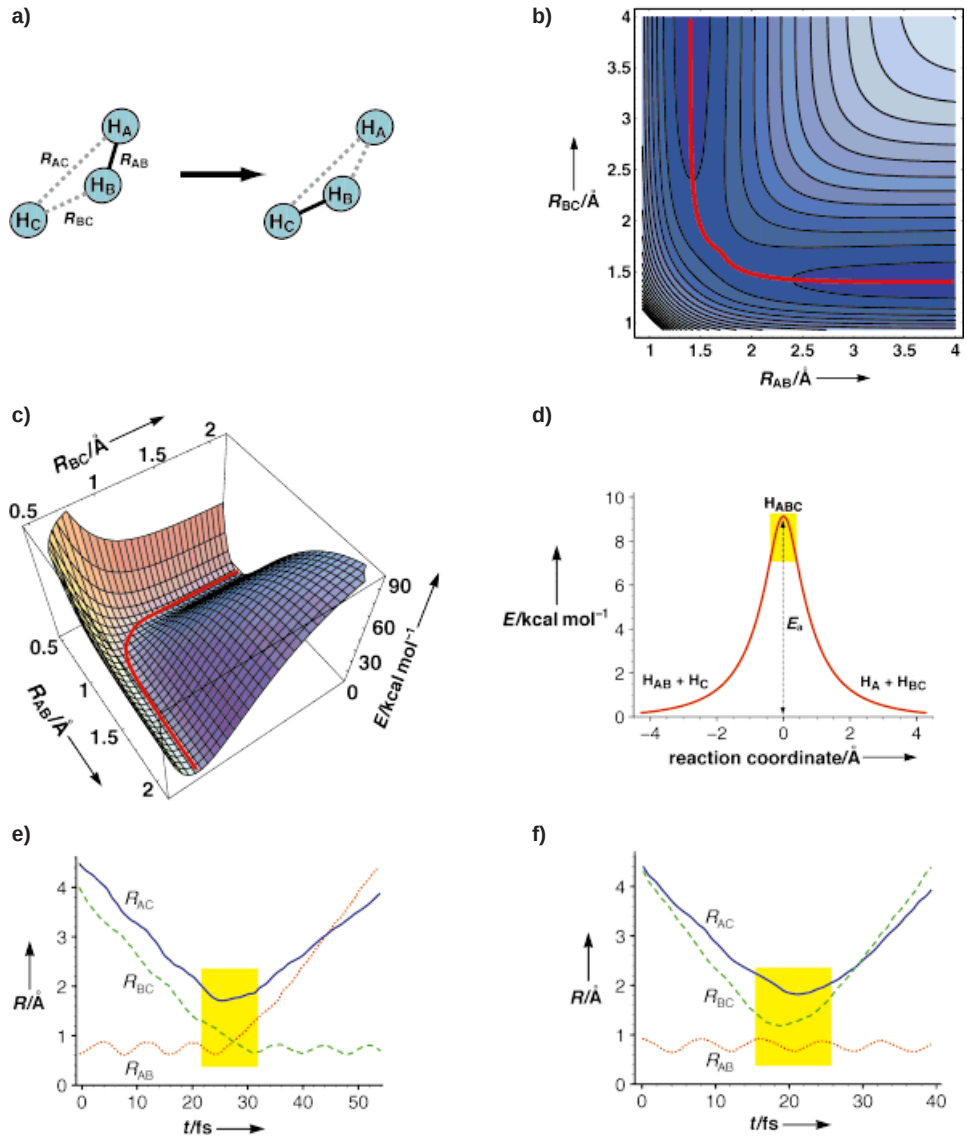


Figure 2. The exchange reaction between a hydrogen atom and a hydrogen molecule. a) Schematic representation of the reaction with definitions for the distances R_{AB}, R_{AC}, and R_{BC}. b) Contour plot of the potential energy surface for a linear collision as a function of the distances R_{AB} and R_{BC} with $R_{AC} = R_{AB} + R_{BC}$; the minimum-energy path is shown in red. c) Same as b), but in a three-dimensional representation. d) Energy along the reaction coordinate corresponding to the minimum-energy path in b) and c); the transition state is indicated in yellow. e) A typical trajectory for the reactive, three-dimensional collision; the three distances R_{AB}, R_{AC}, and R_{BC} are represented as a function of time. f) A typical trajectory for the nonreactive collision; shown as in e). In both e) and f) the interactions between the three atoms are limited to a very short time period (yellow background); this mirrors the narrow potential energy barrier in d). The results are adapted from references [13, 19].

dinates are needed to describe the reaction (Figure 2a). The atoms move on a potential energy surface, which gives the energy of the system as a function of the coordinates. This surface corresponds to the "energy landscape" for the H+H_2 reaction, Figures 2b and 2c show the surface for a collinear collision and the reaction coordinate, which is a combination of the degrees of freedom essential for characterizing the minimum-energy path from reactant to product. The energy along the reaction coordinate is shown in Figure 2d. At a nonzero temperature T the kinetic energies of the H atom and the H_2 molecule are such that the reaction does not follow the minimum-energy path. However, because the valley from reactants to products is narrow and well-defined and there is a highly localized transition state at the top of the barrier, the trajectories remain close to the minimum-energy path at room temperature. As we shall see later, the reaction path for proteins is much less well defined, and the folding polypeptide chain moves over a large region of the energy surface during the folding reaction. This means that, in contrast to H+H_2, the entropy as well as the energy play an important role in protein folding. The well-defined minimum-energy path for H+H_2 results in the simple trajectories shown in Figures 2e and 2f for a reactive and a nonreactive collision, respectively. In the reactive collision an atom approaches, collides with the molecule, and forms a new bond with one of the H atoms, while the other H atom leaves rapidly; the nonreactive collision looks very similar except that there is no exchange of atoms. All of the interesting behavior takes place in only 10 fs

over a very limited range of distances, which is shaded yellow in Figures 2d–f. The initial conditions (positions and velocities) and the interactions in this ultrashort event determine whether the collision is reactive or nonreactive.

3. Protein Folding Reactions

Protein folding reactions have very different characteristics from reactions of small molecules. For example, although the Arrhenius equation can be and often is applied to protein folding, the preexponential factor has a strong temperature dependence.[21] This indicates that protein folding has a complexity not present in most simple reactions. Furthermore, in contrast to the femtosecond time scale of the reactive event in the case of $H+H_2$ (Figure 2), the interactions between the different parts of the polypeptide chain are significant over the entire folding time of milliseconds or longer.

A better analogue for protein folding may be the condensation of atomic clusters (Figure 3), whose thermodynamics and kinetics have been studied in great detail.[22–24] Such clusters have a gas→liquid condensation, which corresponds to the transition from a denatured random coil to a compact but disorganized globule in a protein. If the temperature is low enough, the liquidlike clusters rearrange to an organized solid by a search that is analogous to that through a collapsed globule for the native state in a protein. Of course, a protein has a unique native state determined by its amino acid sequence, whereas clusters composed of identical atoms have many symmetry-related solid structures. In phase transitions of atomic clusters and proteins, multiple pathways are the rule and the transition from the liquid (compact globule) state to the solid (native) ground state is pseudo-first-order; that is, the two states are well-defined and separated by a free energy barrier.

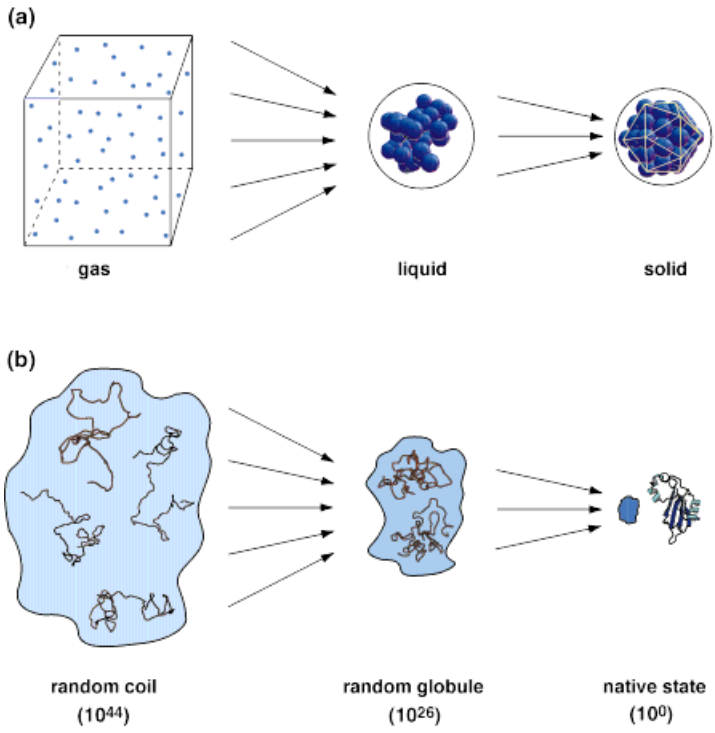


Figure 3. a) Schematic illustration of the phase transitions of a stable cluster consisting of 55 Lennard–Jones particles. The solid cluster has the structure of a Mackay icosahedron.[19] b) Schematic illustration of the corresponding transitions during protein folding. The protein barnase, composed of 110 amino acid residues, is used as an example. Carbon α traces are shown (prepared by the program Molscript).[163] The random coil and disorganized globule conformations were constructed to have radii of gyration of approximately 31 and 15 Å, respectively; the native structure has a radius of gyration of 13.4 Å. The numbers of possible main-chain conformations were calculated from the estimates of the main-chain entropy per residue; there are 2.57 and 1.7 conformations per residue in the random coil and random semicompact states, respectively.[55a] Therefore, the number of random coil states is $2.57^{109} \approx 10^{44}$, and the number of compact random globule states is $1.7^{109} \approx 10^{26}$.

Since proteins and atomic clusters have multidimensional potential energy surfaces involving a large number of degrees of freedom, instead of only three for $H+H_2$, there can be a multitude of local minima and transition regions. Furthermore, the structural changes in folding from the denatured (reactant) state to the native (product) state involve the entire protein, as does the rearrangement to the solid state of a liquid cluster.[22, 23] This suggests that approaches based on the statistical mechanics and dynamics of large systems[2, 25–28] can provide more insights concerning protein folding than the extrapolation from the behavior of small molecules.

An important difference between descriptions of reactions of small molecules and protein folding or cluster condensation is that it is useful to replace the potential energy surface of the former (Figure 2) by a temperature-dependent averaged effective energy surface for the latter. This surface corresponds to a Boltzmann-weighted average of the accessible energies for each value of an appropriately chosen progress variable. The value of the progress variable, which describes the approach to the native state, is obtained by averaging over many nonessential degrees of freedom. The resulting motion on the effective surface is expected to be diffusive.[14] Therefore, it can be described by a Kramers-type model,[29] similar to that appropriate for many solution reactions. An example is the very simple conformational changes from *gauche* to *trans* in liquid butane.[30] Of course, the protein folding reaction is much more complex since a multitude of conformational transitions in a long polypeptide chain are involved. In particular, both protein folding and the formation of solid clusters have a many-body character that plays an important role in the subsequent analysis.

As the comparison with atomic clusters suggests, it is not possible to define a *simple* reaction coordinate on the multidimensional effective energy surface for protein folding. Since there are many ways of reaching the native state, a single minimum-energy path or pathway, in the language of Levinthal,[8] is unlikely to dominate the folding reaction. A progress variable or variables must replace the reaction coordinate in analyses of protein folding. These variables describe the progress of the reaction from the initial to the final state, as does a reaction coordinate, but include the

possibility of many fundamentally different paths. This suggests that our knowledge of folding can never be as detailed as in the case of small molecules, and that we have to be careful in extending concepts from reactions of small molecules, even those catalyzed by enzymes, to protein folding.

If there is no simple reaction coordinate in protein folding, there is also no simple transition state. Indeed, even the rotation of a single side chain in a protein requires an extended region on the potential energy surface (a transition "seam") to describe the reaction.[31] It is likely that the most precise statement possible for the protein folding reaction will refer to a multidimensional transition region on the averaged effective energy surface that includes the structures from which the polypeptide chain folds rapidly and with high probability to the native state. There may be multiple transition regions with similar free energies but significantly different structures that do not interconvert rapidly, in which case parallel paths would contribute to the overall folding reaction. There is also the question of whether standard kinetic schemes, which use the concentrations of a small number of well-defined species, are appropriate for the protein folding reaction.

The temperature dependence of the stability of the various states in cluster formation (protein folding) is determined by the balance between the effective energy and the configurational entropy. The number of available states decreases by many orders of magnitude in the progression from the gas (random coil) through the liquid (compact globule) to the solid (native) state (Figure 3b). This could represent nothing more than the necessary reduction in the number of available states as the system becomes more organized (nativelike). Alternatively, the effective energy function may bias the motion in the direction of the native state and result in a more rapid reduction in the accessible configuration space. The latter is essential to obtain rapid folding of proteins and solidification of clusters. Therefore, the essential problem to be solved is how the system "finds" the ground state or, in other words, what in the nature of the effective energy surface and the resulting balance between energy and entropy determines whether the system can reach the ground state in a reasonable time or not.

4. Thermodynamics of Protein Folding

Prior to describing our current understanding of the folding reaction, it is useful to consider the thermodynamics of proteins. The free energy of the native state of a protein is only slightly lower than that of the denatured state under physiological conditions owing to a near cancellation of large energetic and entropic contributions. The major interactions are the nonbonded van der Waals and electrostatic terms in the potential energy function.[32] They act between the atoms of the protein and between the protein atoms and the solvent. For interpreting folding thermodynamics and dynamics, it is convenient to define an effective energy function that includes one essential part of the free energy of a polypeptide chain in solution. This corresponds to a "potential of mean force"[33] and is obtained from a Boltzmann average at the temperature of interest, of the polypeptide–solvent interactions over all positions of the solvent molecules for a given polypeptide configuration. A potential of mean force of the type considered here can be defined exactly for the system at equilibrium; that is, to describe the thermodynamics.[2] Extending such a potential of mean force to time-dependent phenomena, such as protein folding, requires that the relaxation of the solvent be fast compared to the protein motions involved in the folding reaction. This appears to be a good approximation, as illustrated in simulations of protein unfolding in water.[34, 35] Such an effective energy function, which strongly favors the native state, contains all contributions to the total free energy (e.g., the hydrophobic effect, solvation of polar groups and hydrogen bonding that involve both van der Waals and electrostatic interactions) except the configurational entropy, which strongly favors the denatured state. The latter arises from the fact that the conformation space available to the polypeptide main chain and side chains is much more restricted in the native state than in the denatured state.

Analysis of the contributions to the effective energy (i.e., the potential of mean force) indicates that the nonpolar groups strongly favor the folded state owing to the attractive van der Waals interactions in the tightly packed (solidlike) native structure and the hydrophobic effect, which favors the burial of nonpolar groups.[32, 36] By contrast, the polar groups (the peptide groups and the polar and charged side chains) make a much smaller contribution to the stability of the native state owing to a balance of the interactions in the interior of the protein and those with the solvent. For lysozyme, as an example, calculations show that the nonpolar groups contribute $450\,\mathrm{kcal\,mol^{-1}}$ and the polar groups contribute $87\,\mathrm{kcal\,mol^{-1}}$ at 25 °C to the free energy of denaturation.[36] The stabilization of the native state due to the effective energy term (about $537\,\mathrm{kcal\,mol^{-1}}$) is counterbalanced by a configurational entropy contribution of about $523\,\mathrm{kcal\,mol^{-1}}$ at 25 °C. This yields a net free energy of unfolding of $14\,\mathrm{kcal\,mol^{-1}}$ (on the order of $0.1\,\mathrm{kcal\,mol^{-1}}$ per residue), which is a typical value for globular proteins.[36] In contrast, the energy or enthalpy difference between the native and unfolded state can be significantly larger; for lysozyme at 25 °C, the unfolding enthalpy is $58\,\mathrm{kcal\,mol^{-1}}$.[36, 37]

An important feature of protein folding is that the overall structure, such as that represented in Figure 1, is determined by the sequence of the protein.[5] Native folds for three different proteins are illustrated in Figure 4. The difference in sequence gives rise to differences in the nature of the secondary structure (the regions of α-helix and β-sheet) and the tertiary structure (the overall folding pattern). Three-dimensional structures of approximately 6000 proteins have been determined by X-ray crystallography and NMR spectroscopy.[38] The domains in these proteins can be clustered into approximately 350 families of folds, which consist of sequences that have similar structures.[39] It has been estimated that only 1000 or so different folds exist.[39–41] This is a large number but much smaller (by a factor of about 100 or so) than the total number of different sequences in the human genome. Some folds are adopted by a large number of

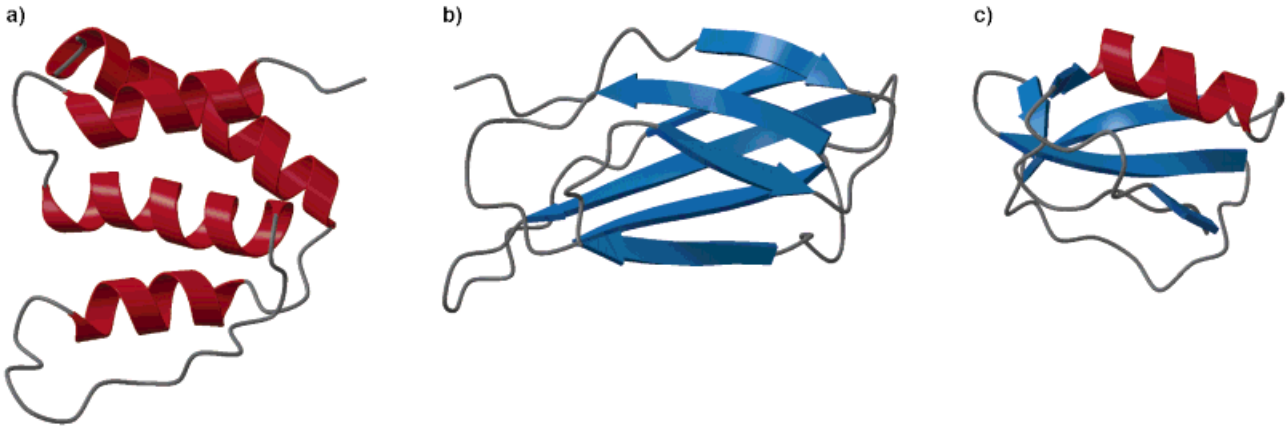


Figure 4. Structures of representative folds (teriary structure) of small proteins. a) Bovine acyl-coenzyme A binding protein (PDB code: 2ABD), an α-helical protein; b) Type-III module of the tenth domain of the human fibronectin (PDB code: 1TTG), a β-sheet protein; c) Barley chymotrypsin inhibitor 2 (PDB code: 2CI2), an α/β protein. Secondary-structure elements correspond to α helices (red) and β strands (blue). The figures were drawn with the programs Molscript[163] and Render.[164]

sequences, which is perhaps related to the stability of the fold[42] or to the robustness and speed of the folding process, but for any given sequence only one fold exists in almost all cases. The uniqueness of the native state arises from the fact that the interactions that stabilize the native structure significantly destabilize alternative folds of the same amino acid sequence; that is, sequences with a deep energy minimum for the native state have been generated through evolution to eliminate wrongly folded or partly unfolded structures at physiological temperatures. This "energy gap" between the native state and significantly misfolded structures[25, 55a] is a key element in the relation between sequence and structure and plays an important role in protein folding, as we describe in Section 5.

To relate the free energy of a protein to its conformations, it is necessary to describe the latter in terms of an order parameter. Qualitative curves of the free energy of the polypeptide chains in equilibrium as a function of the radius of gyration R_g, one possible order parameter for protein folding, are shown in Figure 5 for two different temperatures.[2, 43] At each value of R_g the free energy corresponds to a Boltzmann average over all values of the other internal coordinates, including those of the solvent. The form of the free energy curve is strongly dependent on the environmental conditions, particularly the temperature.

The curve in Figure 5a is valid for a temperature at which the native state is stable. It is significantly below the folding transition temperature, T_m, at which the native and denatured states have equal free energy. The native state is a deep, narrow minimum and thus satisfies the thermodynamic criterion for protein stability. In this minimum the atomic fluctuations have a magnitude of up to approximately 2 Å, and such motions take place on a picosecond to nanosecond time scale.[44] All structures visited by such fluctuations are very similar and correspond approximately to the differences between X-ray structures of proteins that are members of the same family (e.g., myoglobins and hemoglobins from the globin family[44]). Many simulations of molecular dynamics that use empirical potentials have explored this region of conformational space,[45] which is the one most relevant to

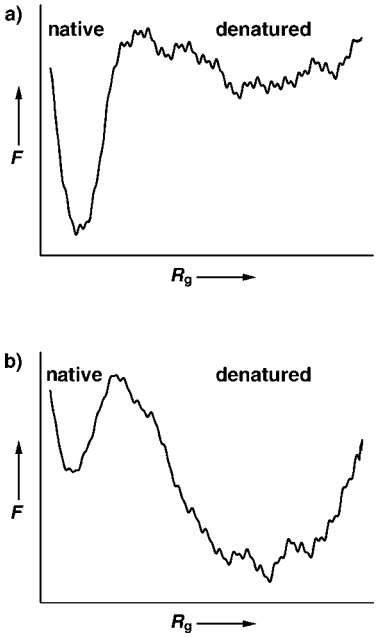


Figure 5. The free energy F of protein folding as a function of the radius of gyration at a) a temperature below T_m, at which the native state is stable, and b) a temperature above T_m, at which the denatured state is stable. The ranges corresponding to the native state and the denatured region are indicated. The curves are schematic but should be representative of real proteins.

protein function. The other region of the free energy curve corresponds to the vast number of configurations which make up the denatured state. In the folding process the polypeptide chain may go from a relatively extended random coil with large R_g to a disorganized compact globule that has liquidlike properties and from there to the native state. The length scale of the structural changes involved is on the order of 10 to 20 Å, and the time scale is in the microsecond to millisecond range. In other cases, the compact globule may lead to an intermediate with some order (e.g. nativelike secondary structure) that is often referred to as a "molten globule".[56] The regions corresponding to a random coil, a compact

globule, and the native state in protein folding have their analogues in the solid, liquid, and gaseous states of atomic clusters (see Figure 3). Phase diagrams for proteins and for simple protein models show the existence of these different phases.[2, 47, 48]

Figure 5b shows the free energy of a protein as a function of R_g at a temperature significantly above T_m. As the temperature is raised, the denatured state is stabilized by its increase in configurational entropy relative to the native state. The curve in Figure 5b also corresponds to the polypeptide chain under denaturing solvent conditions. Although the native minimum may be nearly unchanged, the random coil region has a lower free energy, primarily as a result of its energetic stabilization by better interactions of the unfolded polypeptide chain with the denaturant.[49] Evidence concerning the potential energy surface and the fluctuations characteristic of the different states can be derived from experimental studies; for example, from the rate with which amide group hydrogens in the protein exchange with solvent.[50]

5. Energy Surfaces for Protein Folding: A Theoretical Approach

To determine possible features of the protein folding reaction and the mechanism by which the Levinthal paradox is resolved, it is necessary to go beyond the simple one-dimensional representation used in Figure 5. In contrast to the $H+H_2$ reaction, where a reduction from three variables to one yields a reaction coordinate, a description that is useful for obtaining an understanding of the protein folding reaction requires a reduction from several thousand degrees of freedom to one or a few progress variables. Since the validity of such a reduction is not evident a priori, it must be attempted and justified a posteriori by comparison of theoretical predictions with experimental measurements.

Many phenomenological models for protein folding have been proposed over the years, but most of them were neither specific enough nor detailed enough to permit actual calculations; a recent review of these models is given in reference [51]. The introduction of lattice models with simplified potentials (see Appendix 1) has made possible simulations of folding for systems that include many of the essential complexities of the folding reaction (e.g., there is a Levinthal paradox) and yet are simple enough for a complete analysis (i.e., it is possible to obtain information at any level of detail for the folding process in such model systems). From the lattice simulations[6, 14–16] and statistical mechanical models,[25] insights into possible folding scenarios have been obtained. They provide a basis for exploring the general characteristics of the mechanism of folding for real proteins and for relating experiment to theory. In the following sections, we review the results obtained from lattice models and show what has been learned from them.

Although lattice models (see Appendix 1) are clearly oversimplified,[52] they play a role in the investigation of protein folding that is analogous to that of $H+H_2$ for chemical reactions. The general concepts developed from the detailed analysis of the $H+H_2$ reaction, which involves only three atoms, serve as a basis for the treatment of much more complex reactions with activation barriers. Moreover, the foundations of the theory of the transition state were developed by Wigner[53] for the $H+H_2$ reaction. Simple lattice models treat an amino acid as a single quasiparticle (without representing the side chains), use contact potentials rather than much more detailed potential functions, and restrict the motions to a lattice instead of three-dimensional space. Nevertheless, the detailed analysis of lattice models serves to provide concepts that are useful for understanding the folding reactions of real proteins.

To go beyond the thermodynamic description (Figure 5), it is necessary to find progress variables appropriate for the folding reaction. One possibility is the radius of gyration R_g (Figure 5). However, in some circumstances there is a relatively little change in R_g over the region of interest—for example, if the system goes from a disorganized globule to the native state while remaining compact. A more useful progress variable is the fraction of native contacts Q, which varies from a value near zero for the highly denatured (coil) state to unity for the native state. For the 27-mer, described in Appendix 1, there are 156 different possible contacts and 28 native contacts in a $3 \times 3 \times 3$ cube, whereas for a 125-mer,[54a] there are 3782 possible contacts and 176 native contacts in a $5 \times 5 \times 5$ cube. In a real protein, although there are many more "native contacts", a subset of 50 to 100 contacts can be chosen to define the native state.[54b] This supports the use of the native contacts as a progress variable for the folding reaction.

To visualize the reaction as a function of Q, we use a surface to represent the averaged effective energy and configurational entropy of the polypeptide chain as it folds from the denatured to the native state (Figures 6 and 7). Both quantities are calculated by averaging over a series of simulations for a given sequence. It is this type of average that is suggested by many-body statistical mechanics and by the comparison between protein folding and the formation of solids of rare gases (see Figure 3). The resulting values depend on the temperature of the system; that is, more conformations with higher energies are sampled along the folding trajectory at higher temperatures. Therefore, in drawing and interpreting a diagram, it is necessary to consider the temperature of the simulation.

Figures 6 and 7 show results obtained from folding simulations of a 27-mer lattice model;[30, 55a] this model system is expected to give results applicable to small proteins. Figure 6 corresponds to a low-temperature simulation, and Figure 7 to a high-temperature simulation. For both cases, the native state is stable (in accord with the diagram in Figure 5a), and folding occurs in a reasonable amount of time. At still lower temperatures, folding may be extremely slow because the chain cannot escape from local minima; at very high temperatures the native state is not stable, and the number of accessible conformations is so large that the Levinthal problem cannot be solved. The scenarios illustrated in Figures 6 and 7 could correspond to the behavior of different proteins under the same environmental conditions or to the same protein under different environmental conditions (see below). Since the resulting surfaces are based on statistical

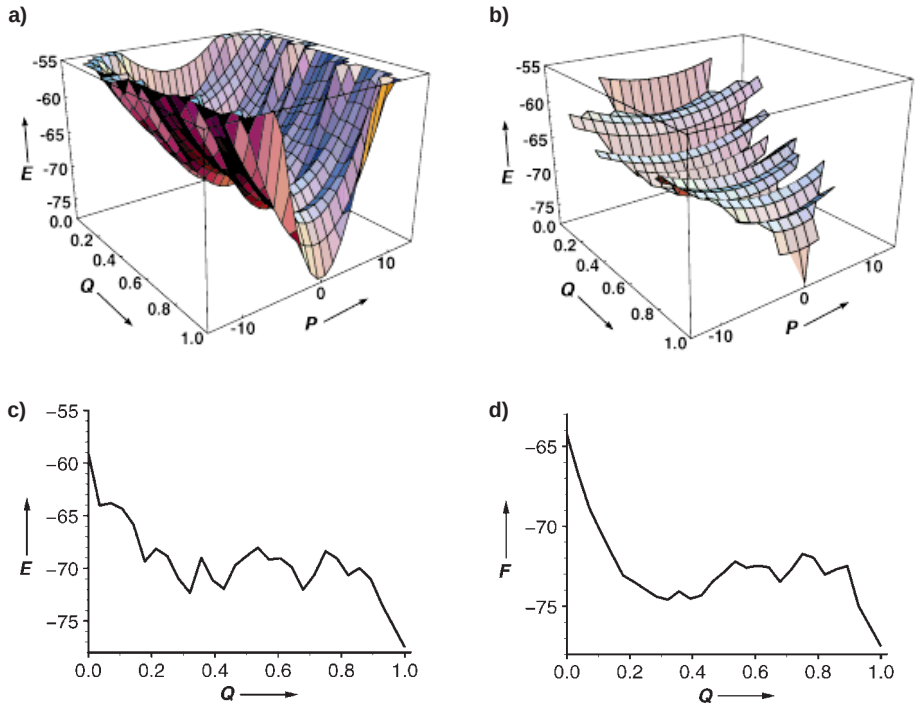


Figure 6. Lattice simulation of the folding of a 27-mer at low temperature. a) The averaged effective energy and the configurational entropy are shown as a function of the progress variable Q (see Appendix 1). At each value of Q, the cross-section of the surface is a parabola whose lowest point corresponds to the averaged effective energy $E(Q)$, and whose steepness is proportional to the configurational entropy $S(Q)$. The function $E(Q,P) = E(Q) + P^2[0.2/(S(Q)+1)]$ is used for illustrative purposes, where P is a measure of the available conformational space. b) The accessible energy surface, the part of the surface which is significantly populated in the reaction, is shown. The surface is obtained by cutting the edges of the surface in a) such that the length of the parabola at each value of Q is $4S(Q)+0.2$. c) The averaged effective energy E as a function of Q. d) The effective free energy F as a function of Q. The results are adapted from the data for sequence 43 in reference [28] for $T = 0.7$ (to relate T to a physical temperature $T = 1$ can be assumed equal to about 250 K.

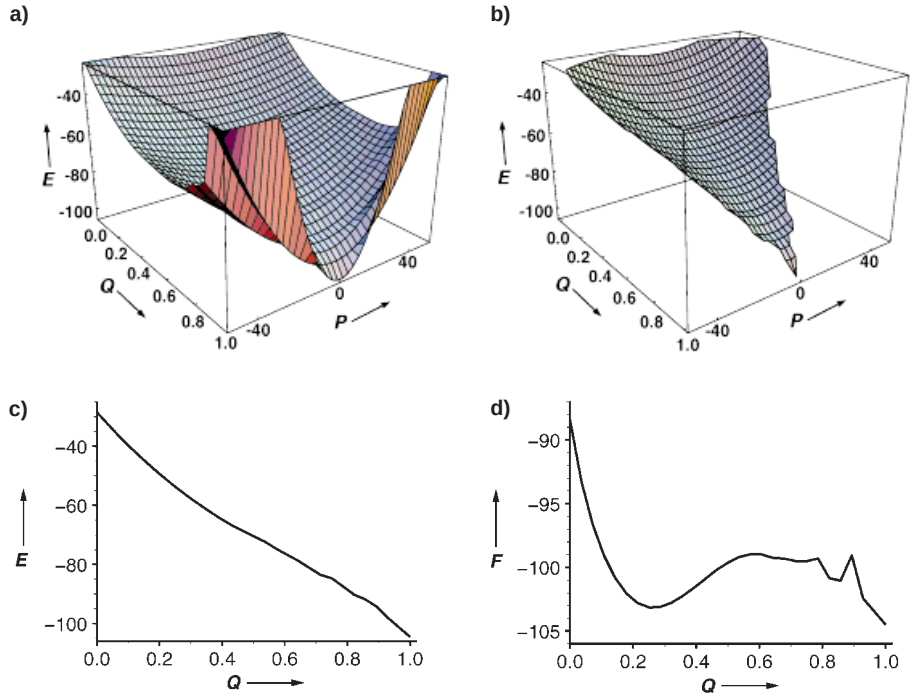


Figure 7. Lattice simulation of the folding of a 27-mer at high temperature. Figure a) – d) correspond to those in Figure 6. The same sequence as in Figure 6 was used except that the native state was stabilized by offsetting the energies of all 28 native contacts by a random number from a Gaussian distribution (mean value: $-0.8\,T$, standard deviation: $0.1\,T$); $T = 2.0$. The spike at $Q = 0.9$ may be a lattice artifact due to the very small number of states.

mechanical averages over the energies sampled in many trajectories, they appear smoother than the actual potential energy variation along individual trajectories, such as the one illustrated in Appendix 1. However, for drawing conclusions about the general behavior of the system, results of the type shown in Figures 6 and 7 are appropriate.

From the low-temperature averaged effective energy/entropy surface shown in Figures 6a and 6b, it is evident that the conformational space accessible to the protein molecules is limited, even at low Q. At the temperature corresponding to that in Figure 6, the polypeptide chain collapses to a disorganized ("misfolded") globule whose Q value is near that of the random coil (see Appendix 1). The configurational entropy at this temperature is sufficiently small that its destabilizing contribution to the free energy on collapse is compensated by the burial of hydrophobic groups, even in the absence of native contacts. As Q increases from 0.2 to 0.8, the average energy does not decrease significantly. In this region, the system makes a slow stochastic search within the collapsed state (see Appendix 1). As is evident from Figure 6c, the averaged effective energy surface is "rough"; that is, there are dips and crests along the valley floor due to the presence of energy barriers to reorganization within the collapsed state. The jagged edges of the accessible energy surface in Figure 6b reflect the "roughness" of the entropy as a function of Q. Intermediates would accumulate if the barriers were high enough or if the temperature was low enough. For the sequence of the 27-mer from which the results shown here were obtained,[28] there was no deviation from simple exponential kinetics in the lattice simulations, even at the lowest temperatures.[56] This indicates that traps do not play an important role; more complex behavior is found for other systems (see below). The transition region in the simulation is close to the native state ($Q \approx 0.7 - 0.9$), as indicated by the free energy profile (Figure 6d).

Furthermore, the simulations show that folding is highly cooperative in that only Q values near the random value of 0.2 and that of the native state ($Q = 1$) are significantly populated during the reaction.[55b]

Figure 7a shows the averaged effective energy/entropy surface at a high folding temperature; the accessible surface is shown in Figure 7b. Early in folding (e.g. for $Q = 0.2$), the surface is very broad, which indicates that the average system is sampling a very wide range of conformations; in fact, most of the random coil configurations that give rise to the Levinthal paradox are accessible. Each individual chain, of course, samples only a small part of the configuration space in moving on the surface. The surface becomes narrower fairly continuously, and the entropy decreases as Q increases to unity for the native structure. The averaged effective energy surface therefore acts as a funnel,[57] in that regardless of the initial position in conformational space, the molecules diffuse downward in energy towards the native state as a consequence of the increase in the number of stabilizing contacts. The steepness of the energy gradient is expected to be correlated with the energy of the native state relative to the average energy of the random coil conformations. This leads to the energy gap criterion mentioned above for the simultaneous optimization of thermodynamic stability and rapid folding.[25, 55a] It is valid for small proteins, as represented by the 27-mer, but more complex folding behavior is found for the 125-mer[54a] (see Section 7).

Despite the smoothness of the effective energy surface (Figure 7b), a transition state barrier in the free energy profile can exist. In simulations of the 27-mer, such a free energy barrier (Figure 7d) arises from a reduction of the chain entropy at large Q; that is, the number of accessible configurations decreases strongly as Q approaches the native state value (Figure 7b). This leads to a "constriction" in the folding funnel that results in an entropy "bottleneck". This can be compared with unimolecular dissociation reactions of small molecules, where a "bottleneck" also exists owing to the requirement that a sufficient fraction of the internal energy must be concentrated in the specific degree of freedom associated with the dissociation process.[1, 58]

For the high-temperature scenario in Figure 7, both the energy and entropy decrease essentially monotonically as the system approaches the native state. Thus, it is the balance between the rate of decrease of the energy and of the entropy that determines if there is a free energy barrier and where it occurs. A different balance between the averaged effective energy and the configurational entropy could move the transition barrier in the free energy to smaller values of Q than that shown in Figure 7d. A simple model based on a smooth funnel with a late barrier was used to obtain a kinetic description that provides additional insights into the folding reaction.[59]

A surface of the general form shown in Figure 7b is likely to result in fast folding, much faster than that expected on the surface shown in Figure 6b. The number of Monte Carlo steps required are on the order of 10^7 for Figure 7 (obtained from simulations) and 10^{10} for Figure 6; the latter is obtained from an extrapolation of Figure 8 (see below), because the Monte Carlo simulations required to obtain an accurate value would use too much computer time. When the order of magnitude arguments in Appendix 1 are applied, this corresponds to 10 ms for the fast folding (Figure 7) and 20 s for the slower folding (Figure 6). Both are in the range of actual folding times for real proteins. Figure 7 represents a simple, but nontrivial funnel; it is simple because the averaged effective energy decreases essentially monotonically in the direction of the native state, and it is nontrivial because the number of states that are accessible to the folding trajectories decreases more rapidly than the reduction calculated from the increase in Q. Folding scenarios based on Figure 7 are intuitive and appealing as a solution to the Levinthal paradox. They correspond to relatively rapid, biased diffusion of the progress variable to the native state; the rate-limiting step is the crossing of an entropic barrier. A description corresponding to a Kramers-type model[31, 60] would be appropriate for the reaction. Such a simple funnel is an idealized representation of one possible type of folding scenario. Lattice simulations have suggested alternative scenarios, like that shown in Figure 6, and more complex scenarios emerge when interpreting experiments or simulations of longer lattice chains (see Section 7). Also important is the fact that the various folding scenarios obtained from the lattice models give no information as to how the averaged effective energy surfaces arise from the sequence and its interactions (see Section 8).

For the scenarios in Figures 6 and 7, the probability of reaching the transition region is small, whether there is an entropic or an energetic barrier. However, it is far greater than in the unbiased random search considered by Levinthal, sufficiently so that these model systems are able to fold within a reasonable time. The essential element, as we have seen, is the bias of the averaged energy surface towards the native state, either by an energy gradient or by a restriction of the accessible conformation space, or both.

An important conclusion from Figures 6 and 7 is that the averaged effective energy and free energy surfaces for a protein have a strong temperature dependence, in contrast to the situation for simple chemical reactions such as $H+H_2$, for which the surfaces are almost independent of temperature. This arises from the minor role of the entropy in most reactions of small molecules and its essential importance in protein folding. There is a striking difference between the temperature dependence of the rate constants calculated for the two types of systems (Figure 8). The rate constant for the reaction $H+H_2$ (Figure 8a)[13] shows Arrhenius behavior, as expected from the reaction coordinate profile in Figure 2d. The reaction is dominated by the activation energy, and the activation entropy plays only a small role; this leads to an activation free energy that is nearly independent of temperature. For the protein folding reaction, the calculated behavior of $\ln k$ versus $1/T$ is shown in Figure 8b.[56] At low temperatures, there is an Arrhenius-like behavior. However, as the temperature increases, the behavior deviates significantly from that expected from the Arrhenius equation: The rate reaches a maximum and then decreases as the temperature increases further.

The calculated behavior shown in Figure 8b has been observed in experimental studies of the temperature dependence of rates of protein folding.[21, 61] Such a curved plot

indicates that the reaction is dominated by energy at low temperatures and by entropy at high temperatures. In both

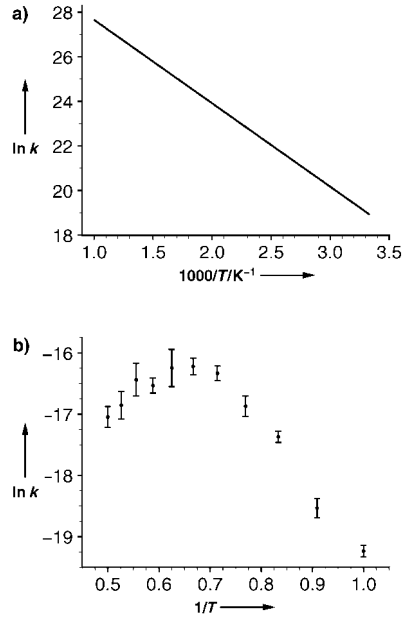


Figure 8. Arrhenius plots of $\ln k$ as a function of $1/T$ (in arbitrary units) for a) the $H+H_2$ reaction (adapted from reference [13]; k has the units $cm^3 mol^{-1} s^{-1}$ here), and b) the protein folding reaction for the same sequence as in Figure 6 (adapted from reference [56]; k is in Monte Carlo steps). To relate T to a physical temperature $T=1$ can be assumed equal to about 250 K.

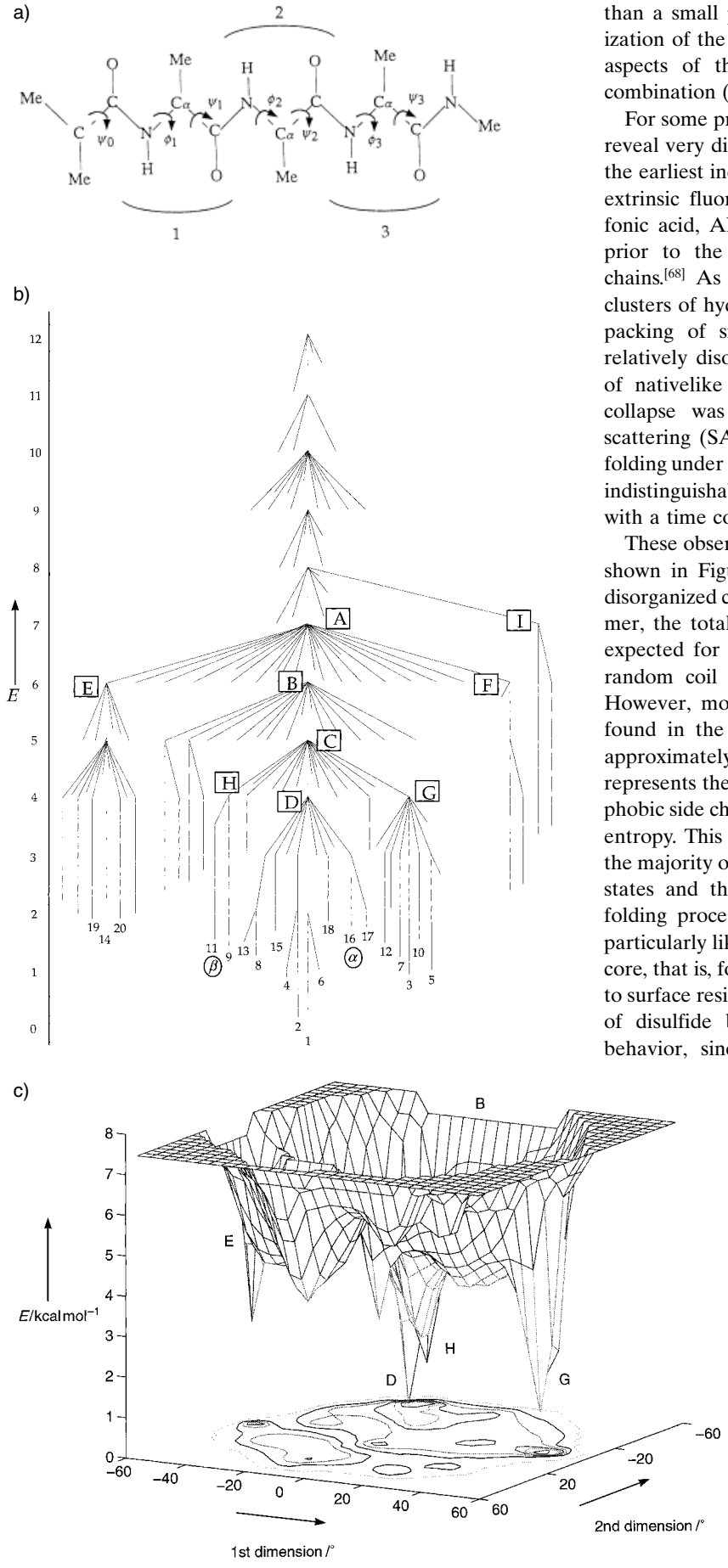
the simulations and experiment, there is a contribution from the activation heat capacity. However, the origin of this contribution may not be the same in the two cases, since the lattice attraction parameter is temperature-independent, whereas the hydrophobic interaction is not.[23] Unfolding, by contrast, is expected to show an Arrhenius-type temperature dependence owing to the large activation energy barrier for escaping from the native state, as shown schematically in Figure 5a. Experiments for lysozyme[61] and barnase[62] demonstrated such simple behavior; the rate constant varies with temperature as depicted in Figure 8a.

The two types of behavior illustrated in Figures 6 and 7 could occur for different systems at different temperatures, depending on the balance of energetic and entropic factors and their variation with temperature, as shown in Figure 8b. For a specific amino acid sequence at a physiological temperature, the observed behavior could be anywhere in the range of possibilities suggested by Figures 6 and 7. Folding scenarios that combine elements of both are likely; that is, funnellike behavior occurs for certain values of the progress variable, and a stochastic search is involved for others. The type of curve shown in Figure 8b for the folding rate constant as a function of temperature is expected to apply to most proteins, although the optimal folding temperature and the maximum folding rate will differ.

The results of lattice simulations point to the essential importance of the energy and free energy surfaces for real proteins. Although a detailed determination of the actual protein surface is beyond present computational possibilities, it is of interest to present here the results obtained for a very small model system: the alanine tetrapeptide described by a detailed atomic model.[63, 64] Figure 9a shows the peptide structure and the important dihedral angles that determine its conformation. In Figure 9b, the complex treelike structure of the multiminimum potential energy surface is indicated. A representation of the energy as a function of the two orthogonal coordinates (principal components) that give the best description is shown in Figure 9c. The basinlike, rather than funnellike, character of the overall surface is evident, as are many deep local minima. The surface is a relatively broad basin down to a certain energy (about 4 $kcal\,mol^{-1}$), at which point the various minima separate into individual basins. Because a protein has a hundred times as many soft dihedral angles as does this small peptide, and because the calculation was done in the absence of solvent (which might be expected to "soften" some of the local minima), it is difficult to extrapolate to the expected behavior for proteins. Nevertheless, the tetrapeptide makes clear that there can be many traps that must be avoided if the sequence is to fold rapidly to the energy minimum corresponding to the native state. How this happens is an essential question that has yet to be resolved, although with a much larger number of degrees of freedom, there will be an increased likelihood of avoiding barriers and traps.

6. Experimental Information on Energy Surfaces

A key objective of experimental studies of any reaction is to obtain information concerning the structures of the species involved as a function of time and to relate this information to the energy surface. Although this has been accomplished in detail only for $H+H_2$ and a few other reactions of small molecules (see Section 2), the insights obtained from studying them have provided a conceptual framework for a wide range of reactions, including most of those important in organic chemistry.[66] In the reactions of organic molecules, it is often true that only a small part of the molecule is actually involved, so that the analysis is not much more complex than for very simple reactions.[67] The problem is much more challenging for proteins because of the very wide range of structures that contribute in all but the final stages of the folding reaction, and because the entire polypeptide chain is involved. This means that it is necessary to determine the distribution function describing the contributing structures as a function of the progress variable. Only in some simple cases, such as those illustrated in Figures 6 and 7, is the time dependence of the *average* structure interpretable in terms of simple kinetic schemes, as in reactions of small molecules. Because of such complications, considerable ingenuity has been needed to devise methods appropriate for structural studies of protein folding (see Appendix 2). Moreover, many folding reactions take place in milliseconds to seconds, which is short compared with the conventional time scales of the techniques available for determining protein structures in solution. Most studies involve a rapid perturbation of the system from equilibrium so that the approach to the native state can be followed. Since no single experimental technique has been able to provide more



than a small part of the detail needed for a full characterization of the folding reaction, methods that probe different aspects of the developing structure have been used in combination (see Appendix 2).

For some proteins, probes for different aspects of structure reveal very different kinetic behavior during folding. One of the earliest indications of this was that for many proteins the extrinsic fluorescence of a dye (8-anilino-1-naphthalinesulfonic acid, ANS) added to the refolding solution develops prior to the intrinsic fluorescence of the aromatic side chains.[68] As the former is indicative of the formation of clusters of hydrophobic residues, and the latter of nativelike packing of side chains, this suggests that collapse to a relatively disorganized globule occurs before the formation of nativelike structure. More direct evidence for such a collapse was recently obtained from small-angle X-ray scattering (SAXS) studies of myoglobin.[69] After 100 ms of folding under acid conditions the radius of gyration is virtually indistinguishable from that of the native state, which is formed with a time constant of about 1 s under the same conditions.

These observations are consistent with the folding scenario shown in Figure 6, in which there is rapid formation of a disorganized collapsed globule. In the simulations with the 27-mer, the total number of contacts is large (≥ 20 of 28), as expected for a collapsed state; this is in contrast with the random coil state, which has seven contacts on average. However, most of these contacts are not those ultimately found in the native structure (the average value of Q is approximately 0.25). Such a disordered collapsed state represents the situation in which nonspecific burial of hydrophobic side chains compensates for the loss of configurational entropy. This can explain the experimental observation that the majority of tertiary interactions are not persistent in these states and that structural rearrangements are part of the folding process.[70, 71] Collapse to a disorganized globule is particularly likely for proteins with a substantial hydrophobic core, that is, for larger proteins for which the ratio of internal to surface residues is high. Other factors, such as the presence of disulfide bonds, may also contribute to this type of behavior, since the formation of cross-links reduces the

Figure 9. The conformation space for a blocked alanine tetrapeptide. a) The peptide and the important backbone dihedral angles ϕ and ψ are shown. b) Tree structure showing the 139 minima of the energy surface of the tetrapeptide; the energies of the minima (indicated by the lower ends of the lines) are given on the scale [kcal mol^{-1}] relative to the minimum energy. c) A simplified three-dimensional diagram of the energy surface of the main basin B, which includes the conformer of minimum energy (basin D), projected onto two principal components [a reduced representation relative to the seven soft dihedral angles specifying the conformation, see a)]. It is interesting to note that the accessible region is rather broad down to an energy of about 4 kcal mol^{-1}, where the different basins separate. Clearly there are many traps on this surface (adapted from references [63, 64]).

entropic penalty for collapse by limiting the disorder of the unfolded polypeptide chain.[72]

For several proteins (including the much-studied α-lactalbumin, which we use as an example) the nature of collapsed, but nonnative, states has been explored in some detail.[46, 73] In the case of α-lactalbumin, the far- and near-UV circular dichroism (CD) signals develop with very different kinetics in stopped-flow experiments (Figure 10b). This indicates that extensive secondary structure exists within a collapsed state prior to the formation of close-packed tertiary structure in the native state.[74] Studies of stable analogues of this kinetically formed collapsed state by a variety of techniques, including measurement of the degree of protection against hydrogen exchange, suggest that the secondary structure has at least some, and possibly extensive, nativelike character.[75, 76] Such secondary structure is not important in the folding of the 27-mer,[28, 55a] but simulations of a 125-mer lattice model (a $5 \times 5 \times 5$ cubic ground state) have shown that the existence of secondary structure speeds up the search in the collapsed state for the nativelike folding "core".[54a] More generally, an appropriate mix of short-range and long-range native contacts appears to be important for fast folding.

Experimental studies on α-lactalbumin have also provided indications of an overall nativelike fold, and of specific tertiary contacts for at least a core within these collapsed (molten) globules[77, 78] (Figure 11), whose important role in folding was originally proposed by Ptitsyn et al.[46, 79] Although

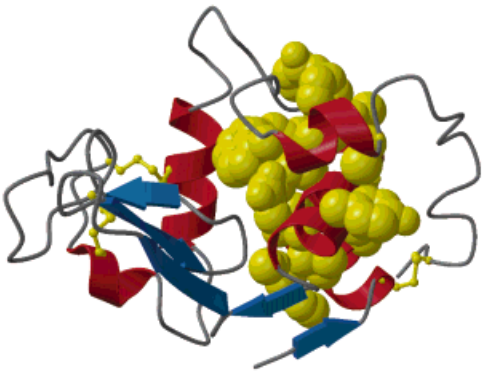


Figure 11. Results of NMR spectroscopic investigations into the protection against hydrogen exchange in the hydrophobic core of the molten globule state of equine lysozyme. The core is formed primarily from amino acid residues that in the native state form three of the four major α helices of the protein (helices A, B, and D); adapted from reference [78]. The α helices are shown in red, the β strands in blue, the disulfide bonds as ball-and-stick representations in yellow, and the side chains of the hydrophobic box core as a space-filling model in yellow; reduced values for the van der Waals radii are used in the latter.

recent experiments suggest that both native and nonnative contacts can exist within the α-lactulbumin molten globule,[80] the former appear to be crucial in defining the nature of the overall fold.[81] Folding of the polypeptide chain to the molten globule can have low cooperativity,[80, 82] while kinetic studies with NMR spectroscopy that monitor individual residues indicate that the close packing characteristic of the native state emerges in a highly cooperative manner.[83, 84] Folding of this type with a late transition state correlates with the simulation results in Figure 6.

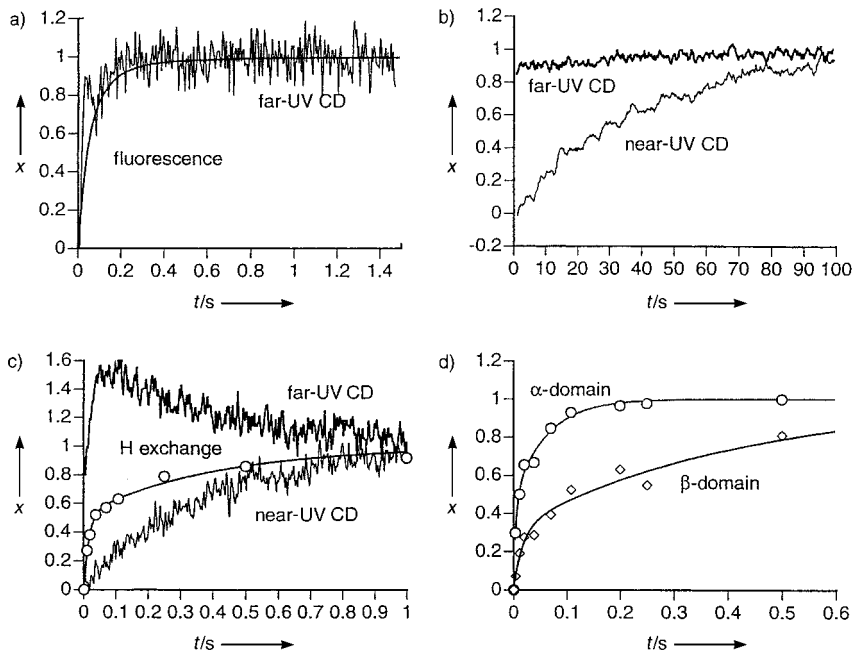


Figure 10. Experimental results from kinetic studies of protein folding. a) Far-UV CD and fluorescence data for the fibronectin domain, ^{10}FN III, shown in Figure 4b (x = fraction of the native signal). All experimental data follow a simple exponential curve.[165] b) Near- and far-UV CD for the apo form of bovine α-lactalbumin.[84] The former (a measure of tertiary structure) develops much more slowly than the latter (a measure of secondary structure). c) Near- and far-UV CD data as well as fraction of hydrogen exchange for hen lysozyme.[98] The overshoot in the far-UV CD indicates the development of nonnative interactions, and the protection against hydrogen exchange prior to the formation of the tertiary structure indicates the presence of intermediates with persistent structure. d) Details of the protection against hydrogen exchange shown in c). The two structural domains of lysozyme form protective structures at different rates, and the fast phase of protection in each indicates that some molecules fold on a "fast track" compared with the remainder of the population.

For some proteins, in contrast to those discussed above, the rate of development of the different aspects of structure, monitored by a variety of physical techniques, is very similar[3] (Figure 10a). This suggests that in these cases there is no significant concentration of partially folded species; that is, there are only small populations of molecules containing some, but not all, secondary-structure elements or displaying secondary structure in the absence of persistent tertiary interactions. A range of proteins with different folding topologies show this type of behavior.[3] The essential common feature appears to be that they are small (ca. 100 residues or less) and do not contain more than one domain in the native structure. The simplest interpretation is that the observed results arise from a kinetic "two-state" reaction; that is, at any given time there is a distribution of molecules consisting mainly of a mixture of "unfolded" polypeptide chains and essentially native ones.

This type of behavior could arise from a funnellike potential energy surface (see Figure 7), provided that there is a sufficient

entropy barrier to obtain single-exponential folding kinetics. The results of lattice simulations shown in Figure 7 yielded an entropic barrier that occurs late (at high Q). However, as already mentioned, an early barrier (at low Q) is also possible; in the latter case, only a small fraction of the native contacts would exist in the transition region. This would result in a "nucleation" mechanism, in which formation of a nucleus involving specific contacts of a small set of residues is the rate-determining step of the folding reaction.[85, 86] The free energy of the system begins to decrease only when the energetic stabilization from nativelike contacts is sufficient to overbalance the entropy loss due to the reduction in accessible conformations when the nucleus is formed; a detailed description of nucleation in sickle cell fiber formation by hemoglobin is of interest in this regard.[87] Simulations of lattice models obtained with sequences that maximize the energy gap between the ground state and the average energy of a random globule fold rapidly by a nucleation mechanism whose transition region corresponds to a Q value of about 0.2.[16, 86] Such a nucleus can still have a large entropy because many configurations are consistent with its existence. A nucleation mechanism has also been proposed for the folding of small proteins on the basis of experimental studies (see below).[88] There appears to be no case where the experimental data on the millisecond time scale are indicative of a gradual accumulation of structure in a small protein, as would be expected for a funnel without a free energy barrier.

For the nucleation-based folding mechanism, the specific structure of the nucleus, which is far from that of the native state, is of particular interest. A powerful approach for investigating this (transition state) region is based on protein engineering.[89] It involves mutating specific residues and measuring the change in the rates of folding and unfolding relative to the effect on the overall stability of the protein. As an example, if a residue has nativelike contacts in the transition region, a mutation would be expected to have approximately the same effect on the stability of the transition region as on that of the native state. Consequently, the mutation would perturb the folding rate but not the unfolding rate; if a residue has no transition-region contacts, the inverse would be true. By studying a large number of residue mutations in this way, a detailed characterization of the transition region is possible.[89] The method has been applied to several proteins, including the barley chymotrypsin inhibitor CI2,[90, 91] a fast-folding protein with two-state folding kinetics of the type discussed above. These experiments show that a small group of residues has nativelike contacts in the transition region. This set of residues is highly conserved in a sequence optimization simulation of CI2 and in a series of related sequences.[92] The results are suggestive of a broad transition region rather than a well-defined transition state, because an ensemble of species with very different structures can have the same or a similar nucleus.[93] A recent study of the folding kinetics of two forms of cytochrome c, which have very similar native structures but only 60 % sequence identity, also suggests an early transition region in which only the conserved regions have a well-defined structure.[54b, 94]

Although the two experimental scenarios for folding described in this section appear to be very different, they have a common feature: The folding process involves a search of conformational space for nativelike contacts that bring together residues in space. The difference in the averaged effective energy surface and the resulting kinetics reflects the importance of the contacts between specific residues. For certain sequences, a collapsed state is energetically favored over extended states, even if most of the contacts involve residues of only the appropriate type (e.g., those with hydrophobic side chains). In the case of folding on such a surface, the conformational search takes place within the collapsed state, and the reorganization of contacts to form the late transition region may involve overcoming significant barriers that would not be present in more expanded states. For other proteins, a collapsed structure is stable only with a set of highly specific and energetically favorable (nativelike) contacts. In this case, the search for a rather small nucleus can take place mainly in the unfolded state, and the transition region is early in the folding process. Mutations and/or changes in the environment can alter the averaged effective energy surface and change the details of the folding reaction by altering the balance between the effective energy and configurational entropy; this may result in a shift of the transition region. This effect has been observed experimentally in several proteins.[95, 96] Significant changes in the position of the transition region, which indicates its labile character and its critical dependence on the balance between the effective energy and entropy, have been found for a series of Ala $\rightarrow$Gly mutants (in which Ala was replaced by Gly) of a monomeric form of the fast folding λ-repressor protein.[97]

7. Complexities in Folding

Many reactions of small molecules have more complex kinetics than the $H+H_2$ reaction. There can be a series of intermediates and more than one product. The reaction-rate theory can readily be extended to include such features. Correspondingly for proteins, particularly larger ones, more complex behavior has been observed experimentally than for the cases discussed in the previous section. An approach to understanding their folding is to use the knowledge gained from simulations of the simpler systems to propose averaged effective energy surfaces that are consistent with the experimentally observed properties.

A schematic surface for a complex folding scenario is shown in Figure 12. It is based primarily on experiments with lysozyme and is generated so as to correspond to the results shown in Figures 10c and 10d. In particular, the surface incorporates features which account for two of the complexities observed in the folding process.[69, 98–101] The first of these is that distinct intermediate species accumulate during folding. This indicates that there are significant minima along the effective energy surface prior to the transition region for the formation of the native state. The second is that the native state appears more rapidly for some of the refolding molecules than for the remainder.[100, 101] This suggests that there are fast and slow folding trajectories and that a "ridge" in the energy surface inhibits exchange between them. Such results can be related to the mechanism of "kinetic partitioning" discussed by Guo

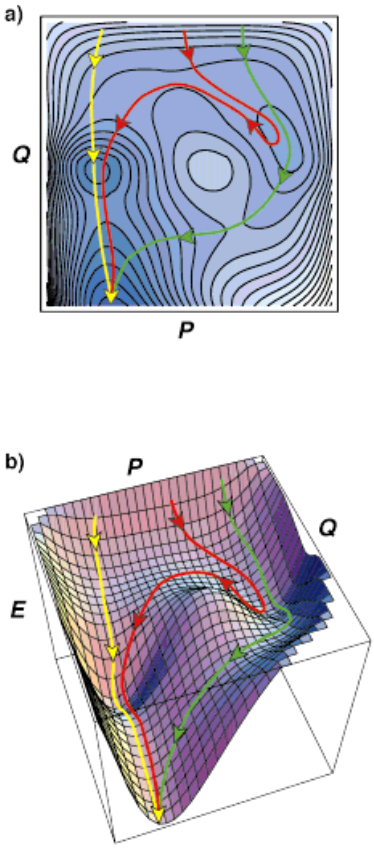


Figure 12. Averaged effective energy surface based on experimental data for the folding of lysozyme (see text, Appendix 2, and Figure 10) illustrated as a) a contour plot and b) a three-dimensional surface. Several possible folding trajectories are shown: a trajectory for fast folding (yellow); a trajectory for slow folding that crosses the high energy barrier (green); a trajectory for slow folding (red) that returns to a less folded state and then follows the valley corresponding to the trajectory for fast folding.

and Thirumalai.[102] Incorporating such behavior in an effective energy surface requires that the progress of the reaction be specified by more than the single native contact variable Q, which we have used for the simpler surfaces described above. At least one additional variable is required. It is indicative of our relative ignorance about the detailed mechanism of folding that there is not enough information to assign a specific physical meaning to the additional variable. It could, for example, involve the number of nonnative contacts in a partly folded state.[54c] This gives a measure of compactness, as would the exposed surface area.

Intermediates arise in the folding of lysozyme because persistent structure does not form cooperatively for the entire protein from the collapsed state that is generated early in the folding reaction; this collapse is represented by the steep drop at the top of Figure 12. Instead, nativelike structure develops independently in the two distinct domains that make up the native fold (see Figure 1); one domain is largely α-helical, and the other contains a substantial region of β sheet.[103] As one domain (the α-domain) folds faster than the other, a partially folded state with structure in the faster folding domain will result. In the surface of Figure 12 this corresponds to the deep minimum on the right-hand side. Within each domain the formation of persistent structure is largely cooperative, and can, but does not always (see below), take place on a time scale similar to that for the complete folding of a small, single-domain protein.[99, 101] The two domains coalesce in a slower step which is coupled to the formation of fully native character within the domains; this is indicated by the barrier on the left-hand side of Figure 12 along the yellow trajectory. In accord with these experiments, more than one folding domain was found in lattice simulations of longer chains.[54a, 104] The results suggest that domains[105] are likely to be an important feature of the folding of larger proteins, in contrast to small proteins (and short chains on lattices) in which the only cooperative unit may be the entire polypeptide chain.[3, 89]

There is abundant evidence that intermediates are common in the folding of all but the smallest of proteins. Important information concerning them can come from the results of protein-engineering experiments of the type discussed above for mapping the transition region. One of the larger proteins for which such studies exist is barnase, a 110-residue ribonuclease from *Bacillus amyloliquefaciens*.[89, 106] Like lysozyme, this protein forms a well-defined intermediate, in which substantial nativelike structure exists. The protein-engineering experiments show that additional nativelike structure is present in the transition region for folding, which occurs at significantly higher Q values than for small proteins such as CI2. As in lysozyme, the rate-determining step may involve the docking of partially preformed domains[89]

The observation of kinetic intermediates in the folding of larger proteins, including lysozyme and barnase, has prompted considerable discussion as to their significance.[76, 107–109] There are two extreme views. One is that these intermediates are productive and help to direct the folding process towards the native state by acting as templates for the formation of nativelike structure and restricting the conformations that need to be sampled. This can speed up the search and, once again, differentiates protein folding from reactions of small molecules, in which intermediates generally decrease the reaction rate. Alternatively, intermediates could represent kinetic traps that, for example, involve misfolded species which hinder the folding process and slow down formation of the native state; such intermediates appear in the lattice simulations of the 125-mer.[54a] This is the situation found for reactions of small molecules, in which an intermediate often decreases the rate, although it may play a crucial role in determining the product. It is likely that in protein folding both types of intermediates occur and that their role can depend on the amino acid sequence and the environmental conditions, as indicated by experiments on ubiquitin[96] and cytochrome c.[110]

The existence of both fast- and slow-folding molecules in lysozyme also sheds light on the role of intermediates in folding. In some systems distinct populations that fold with different rates are associated with isomerization of the protein backbone.[111] In particular, in an unfolded polypeptide chain *cis* and *trans* isomers of proline residues have comparable stability, and the barrier to their interconversion is large; the folding reaction for the molecules which exist in the nonnative isomeric state at the onset of folding therefore includes an additional slow step. In the case of lysozyme, however, the

origin of the kinetic heterogeneity does not appear to be associated with proline residues.[70, 100] The existence of parallel folding routes instead suggests that species with different conformations are generated relatively early in the folding reaction, and that significant barriers to the interconversion between the slow- and fast-folding populations exist; the slow-folding species have to go over a higher barrier to reach the native state or to unfold and return to the fast pathway (see Figure 12). The experimental studies indicate that the intermediate state populated on the slow -folding trajectories has elements of the nativelike structure within one of the two domains of the overall fold, whereas that populated on the fast-folding trajectory has such structures within both domains.[99, 101]

These findings imply that the rate-limiting step in the folding process for the slow-folding population of lysozyme molecules involves, at least to some extent, the reorganization of misfolded species.[70] As the collapsed states in lysozyme have substantial secondary structure and four disulfide bridges, it is not unreasonable that such rearrangement processes are subject to significant barriers and hence observable in experimental studies. The occurrence of this type of behavior is supported by the results of lattice simulations. In the collapsed globule seen in the 27-mer simulations, there are many nonnative contacts; that is, the number of contacts N is much larger than Q (see Appendix 1). Although the resulting species are not stable enough to be intermediates, they are manifest in a roughness of the effective energy surface for the low-temperature simulations (see Figure 6c). The energy surface is such that the polypeptide chain does not have to expand significantly to escape from the minima and progress toward the native state. Trajectories in which misfolded species are more important have been observed in lattice simulations of 125-mers.[54a,c] In these simulations, as already mentioned, a folding core or nucleus is formed early in the reaction. It consists of about 30 residues and has significant secondary structure. The folding trajectories show populations in which the remainder of the polypeptide chain condenses on this core and directly forms the native state, and others in which misfolded but relatively stable structures are formed that slow down the folding reaction. In the case of the latter, a slow step involving significant unfolding is required before reaching the native state; that is, Q is reduced substantially before it can again increase. This occurs, for example, for the trajectories in which two parts of the system have developed nativelike structure but have incorrect relative orientations. The results of the 125-mer simulations have much in common with the experimental data for lysozyme and also with recent results for cytochrome c.[96, 112]

8. A Unified View of Protein Folding

It is useful at this stage to present the general picture of protein folding that is beginning to emerge from heteropolymer theory, from lattice simulations of simplified models, and from experiments on real proteins; we do not repeat references in this section, which unifies the ideas and results presented earlier. When a polypeptide chain is placed under refolding conditions, the van der Waals and electrostatic interactions within the protein and between the protein and solvent stabilize the native state. However, the greater stability of the native state relative to the denatured state does not by itself explain how the polypeptide chain finds the former (a single state out of an astronomically large number of denatured configurations) starting from the latter. The essential element, as illustrated by the lattice simulations, is that the search is not random, but is significantly restricted by the fact that only a portion of the averaged effective energy surface is accessible (see Figures 6 and 7); that is, certain regions are too high in energy to be sampled with a significant probability by folding trajectories. Such restrictions on the space to be searched and an overall bias of the energy toward the native state mean that the polypeptide chain never faces the problem posed in the Levinthal paradox.

In one scenario of protein folding, the polypeptide chain collapses rapidly to a compact globule. Although the most significant factor leading to such an early collapse is the burial of hydrophobic groups, the nature of the collapse depends on the heterogeneity of the stabilizing interactions. The collapse can lead to a disorganized globule without specific contacts and with a relatively large configurational entropy, or to a compact structure with some highly probable contacts and a lower configurational entropy. In the former case, the collapse does not involve a search (i.e., contacts can be formed in any order), and it can be fast. Fluctuations within the collapsed state bring different groups into contact with each other, and the search for the critical contacts that are required to reach the transition region is the slow step. This type of folding has many elements that correspond to those associated with the effective energy and free energy surface shown in Figure 6.

Under other circumstances, chain collapse does not lead to a decrease in free energy unless specific contacts are formed. The search process that brings the required residues together can be aided by intrinsic structural preferences and the formation of hydrophobic clusters that exist even in the highly unfolded state. These rudimentary structural elements can be further stabilized by the formation of main-chain hydrogen bonds (e.g., as in neighboring β strands or in α helices), so that larger regions of structure are formed in a synergistic manner. Transient fluctuations of such nascent structures within a noncompact state lead to collapse only when the required stabilizing nativelike contacts are present. They constitute a "folding core" or "nucleus", about which the rest of the structure can coalesce. The slow step corresponds to the search for the core structure from which the system proceeds rapidly to the native state. There is a relatively early transition region, and proteins with such a free energy surface are likely to fold fast and in an apparently two-state manner. The less stable the native state is, the more nativelike contacts are needed for the compact molten globule state to be formed, and the slower is the folding process.

As proteins increase in size the scenarios become more complex, though the nature of the interactions remain the same and the folding mechanisms build on the elements described for smaller systems. The stochastic search or nucleation mechanism can involve finding a relatively local

folding core that is the size of a small protein, and additional restrictions on the search are required to permit folding in a reasonable time. These can be based, in part, on the chain preferences that already exist in the random coil state.[113, 114] Under native conditions, short-range interactions can give rise to structural features (e.g. β turns or nascent helices) which act as initiation sites to increase the probability of coalescing to structures involving longer range contacts. In many cases, incorrect structures will form that need to be undone. At the early stages of folding this is likely to be fast, but later in folding significant barriers to the rearrangement of misfolded elements may lead to slow steps in the folding process. High stability is important for formation of the core, so that it exists long enough for condensation of the rest of the polypeptide chain to be able to take place. Too much stability in noncore regions can lead to intermediates, some of which may be misfolded. Populations of folding chains may access different regions of conformational space. Therefore, they have to surmount different barriers, giving rise to heterogeneity in the folding kinetics. This type of folding, including complex kinetics, has been found in lattice simulations of larger proteins as well as in experiments. It is likely to occur when there exist relatively structured portions of the protein distant in the sequence that have to be brought together to form the native state.

From the above it follows that—although the events in the folding of different proteins, or of the same protein under different conditions, may appear to be very different—the underlying elements of the folding mechanism are the same. Folding is a progression in which nonnative and native contacts, some of which may be particularly important, stabilize nativelike features of the structure. The energy penalty for nonnative interactions, or misfolding, increases throughout the folding process. The polypeptide chain is directed by stabilizing interactions into productive regions of conformation space that permit an effective search for the native state. Although energy biases restricting the configuration space are essential for folding within a reasonable time, a continuous funnellike gradient in effective energy is not required and appears unlikely to exist in real proteins. A more "stepwise" behavior, involving the sampling of regions that are downhill in energy and other regions that are relatively constant (or even uphill) in energy on the effective energy surface, appears probable. Analogous behavior was observed in folding simulations of clusters of rare gases.[23] The trajectories followed by individual polypeptide chains are likely to be very different, at least initially, with an increasing number of common features as the system approaches the native state. It is only the ensemble of individual molecule trajectories which sample the full accessible configuration space.

To complete the above description of the folding process requires an understanding of how it is encoded in the sequence. It is the sequence which determines the energy surface of each particular protein. Such information cannot be obtained directly from the available lattice simulations. The simulations that have been used to explore the full conformation space are oversimplified and do not contain sufficient detail to relate the specific results to those for individual proteins. Nevertheless, the present analysis of the folding mechanism gives clues as to how folding might encode. An essential aspect of the process is that the specific nature of individual residues becomes more important as the polypeptide chain approaches the native state. The local interactions in the unfolded state lead to a significant probability for the formation of nascent nativelike helices and strands within the ensemble of rapidly interconverting conformers.[113, 114] As a folding polypeptide chain undergoes a collapse transition, the nonrandom distribution of residues of different character (e.g. those having hydrophobic or hydrophilic side chains) generates an architecture, or global fold, that has the general characteristics of the native state. However, at this stage of folding, which often results in a relatively disorganized globule, the specific nature of the residues is of little importance, and very different sequences with similar characteristics can generate the same overall fold.

In defining such an architecture, contacts between some residues are more important than others in bringing together regions of the polypeptide chain which are not close to each other in the sequence. Another essential aspect of finding the correct fold is the need to avoid highly destabilizing situations, such as an unbalanced charged residue in the protein interior. For the final assembly of the native state, the specific interactions in all parts of the sequence become important. The formation of the closely packed structure optimizes the van der Waals and other stabilizing interactions. Even one residue in a nonnative environment can significantly destabilize the structure (e.g., a side chain that introduces a large cavity in the core), though a plasticity towards the replacement of certain residues by others does exist.[115, 116] Because the native state is in a deep energy well, most of the polypeptide chains reach this state and remain there under folding conditions. These physical aspects of the thermodynamics and kinetics of protein folding have their analogue in the use of threading algorithms for identifying the structure associated with a given sequence.[9, 117–122]

The packing of side chains is unlikely to be involved in the resolution of the Levinthal paradox, and side chains are not included in the lattice models used in developing the concepts presented here. However, this packing may be essential for a first-order transition between the deep energy minimum associated with the native structure and the large population of loosely packed globules with backbone structures that are similar to the native state. Indeed, in attempts to design small proteins, it appears to be relatively easy to obtain a compact globule with the correct topology (e.g., a four-helix bundle).[123, 124] The difficult part is to modify such a globule so as to introduce the full nativelike properties that arise from the solidlike packing of side chains. The sequence of a real protein generates the deep native state minimum by a specificity that goes beyond that of the simple patterns of hydrophobic and hydrophilic side chains; the sequence permits specific residues to pack correctly to form the unique native structure. Such packing must be strong enough to overcome the additional entropy loss involved in going from the multiconformational molten globule to the unique native state. The final stage in folding thus involves the cooperative generation of highly compact structures in which specific side chain packing is likely to play a critical role.

9. Summary and Outlook

Detailed calculations for the $H+H_2$ reaction and the concept of reaction surfaces revolutionized the fundamental understanding of chemical reactions. The "new view",[107, 125, 126] which is based on averaged effective energy surfaces[28] or energy "landscapes",[14, 17, 127] plays a similar role in our understanding of protein folding. The new view has emerged from theoretical models and lattice simulations. The latter have played an essential role because they capture many of the complexities of protein folding, but are still sufficiently simple to permit detailed studies of the folding process.[6, 52] There is an essential difference in the new view from that of Levinthal presented in Section 1. The astronomically large number of states that exist for the denatured protein has to be searched in a random fashion to find the native state only if, with the exception of the native state, they all have essentially the same energies. This has been called the "golf-course model" of the energy surface.[25, 55a] As the simulations of lattice folding of the 27-mer demonstrate, even a set of random sequences contains a significant fraction that fold rapidly.[55a] The search through the denatured configurations is highly restricted because only a relatively small subset has energies low enough to be sampled at the given temperature. In addition, the search may be guided by the decrease in energy in the direction of the native state. This leads to a range of folding scenarios, in all of which there are many ways of going from the extended coil to the unique native state. The resulting multiplicity of folding populations requires that reduced progress variables on an averaged effective energy surface be used to describe protein folding as a replacement for the reaction coordinate on a potential energy surface for small molecules. The appropriate progress variable or variables must capture the essential features of the folding reaction and be accessible to experimental investigation. One type of progress variable (the fraction of nativelike contacts) has been shown to be sufficient for simple surfaces. However, as demonstrated in the analysis of lysozyme folding, additional variables are required for more complex systems. Furthermore, it has become clear that to reach an understanding of the underlying principles governing the folding reaction, the averaged effective energy and the configurational entropy need to be considered in addition to the total free energy. This is essential, for example, for an analysis of the non-Arrhenius behavior of protein folding as a function of temperature.

As a consequence of the large number of significant degrees of freedom, the transition "state" is much more complex than in reactions of small molecules. Lattice simulations suggest that there is likely to be a transition region or regions with many configurations that lead rapidly to the native state. If the transition region is late, it consists of an ensemble of structures that resemble the native state, and the interesting step involved in the resolution of the Levinthal paradox concerns the mechanism by which it is found. If the transition region is early in the folding process, it can involve a wide range of very different structures with only a small portion of the structure being well-defined. Knowledge of that portion of structure is then crucial for understanding the folding process.

An important result from the lattice studies is that the difference in behavior observed experimentally between proteins that fold rapidly (and without intermediates) from the extended coil state and those that pass through collapsed states and kinetic traps does not require a qualitative difference in the averaged effective energy surface. Instead, the differences in folding behavior can result from a change in the balance of the configurational entropy and the averaged effective energy as a function of the progress variable. It is possible experimentally to change from one type of behavior to another, for example by mutagenesis to incorporate more hydrophobic groups in the core of the protein (see Section 6).[96] In simulations, a change in behavior can be produced by reducing the temperature to decrease the entropic term (see Figures 6 and 7), and by increasing the attractive hydrophobic interaction between the amino acids.[128]

The multiplicity of ways of reaching the native state suggests that the use of simple kinetic schemes for the analysis of the protein folding reaction has to be reevaluated. As long as measurements on a certain time scale (usually milliseconds or longer) yield single-exponential behavior or the results can be described by a few exponential terms, a focus on "chemical" species is a useful way to summarize the reaction. However, the species may not represent the conformationally well-defined entities of reactions of small molecules, but may instead correspond to a broad distribution of structures. A simple scheme is appropriate if there is a separation of time scales; that is, the equilibration of the conformers corresponding to a given chemical species is fast relative to the interconversion of different species. For example, in the folding simulations corresponding to Figures 6 and 7, single-exponential kinetics are observed. This can be described by the standard scheme $U \rightarrow N$, in which the unfolded species U includes the random coil and collapsed globule region (10^{16} states in the 27-mer), and there is a single native state N. The collapse to a globule, which is an important part of the folding process, does not appear in the simplest kinetic scheme because it is fast and does not affect the overall reaction rate. Therefore, even if a simple kinetic model is able to describe the available data, this does not mean that the folding reaction is understood. The results of simulations and their analysis indicate that there are "hidden" complexities in the protein folding process; that is, proteins that fold with a "two-state" transition are likely to involve a multistage process if probed on a shorter time scale and exhibit more complex behavior on this time scale. This is analogous to the increase in the observed complexity with improvements in the experimental techniques found in other apparently simple reactions in proteins, such as ligand rebinding after photodissociation in myoglobin.[129]

Experiments that make it possible to study protein folding at shorter times and to higher structural resolution are clearly needed.[130] Such techniques are being implemented at the present time, and some preliminary results are already available. Of major importance is NMR spectroscopy, which is beginning to make it possible to study folding in real time.[84, 131, 132] Recent developments include the introduction of novel two-dimensional methods to study folding at the

level of individual residues[83] and the ability to observe at least some aspects of the folding process on a time scale of tens of milliseconds.[133] In addition, measurements of the nuclear Overhauser effect (nOe) to determine intramolecular distances can probe the development of native and nonnative contacts during folding, a crucial aspect of more detailed comparisons with simulations.[80] Other spectroscopic techniques that give specific structural information, such as IR[134] and vibrational Raman spectroscopy,[135] are also beginning to be applied to protein folding. Studies of the larger fluctuations in proteins at equilibrium, as probed by amide hydrogen exchange and other techniques, are being related to the effective energy surface for folding and unfolding.[136, 137]

Methods are currently being developed to probe the very early events in folding by using fast mixing devices or rapid initiation methods on the micro- to nanosecond time scale[138–142] Studies of unfolding[134] in the submicrosecond range are of interest as a complement to the examination of the folding process. Furthermore, such unfolding experiments can be compared directly with detailed all-atom simulations of unfolding initiated by high temperatures or low pH.[2, 34, 35, 143–145] Simultaneously with the experimental developments, theoretical approaches are being improved to permit more realistic studies of folding. These include, for example, all-atom calculations with an implicit model for the solvent,[54b] lattice models with explicit representation of the side chains,[146] simplified off-lattice models of polypeptide-like chains,[147] and the extension of methods for determining averaged effective energy and configurational entropy surfaces for the folding process to all-atom models of the protein and the solvent.[148] The continued increase in computer power is critical to progress in this area.

At their present level of detail, lattice simulations cannot determine the folding mechanism for any specific protein. The utility of the simulations is to suggest general concepts and possible mechanisms. Experiments are essential to show which, if any, of the proposed folding scenarios are correct and to suggest refinements of the models. Some tests of the conclusions from lattice simulations have been published already.[6, 88, 149] At the present stage our knowledge is not sufficient, however, to demonstrate that any protein has an effective energy surface that corresponds to a simple "funnel".[57] We indicated above that such a surface is unlikely to exist, if for no other reason than that there is no evolutionary pressure to create an idealized world for the theoretician. Nevertheless, the limitation of the available experimental data are such that qualitative interpretations in terms of funnels are adequate to describe a number of the folding studies[93] In this review, we have suggested how simulations and experiments can be used to go beyond such descriptions. It is necessary to first map out an approximate effective energy surface and its structural correlates, to describe the dynamics of folding on that surface, and to determine how the structure develops with time. Comparison of the results with more detailed structural information on shorter time scales from experiments can be used to improve the model and to serve as the basis for simulating more realistic models. This makes it likely that an understanding of the basic principles of the folding of real proteins will be achieved in the next few years.

The fundamental principles of protein folding have practical applications in the exploitation of advances in genomic research[150] and in the design of novel proteins with special functions.[151] In addition, it is important to underline that a process as critical for a living system as protein folding is aided by a sophisticated series of helper molecules. Enzymes speed up potentially slow events in folding such as proline isomerism and disulfide bond formation.[4, 111] Moreover, molecular chaperones serve to avoid intermolecular interactions in cells which would otherwise lead to aggregation in such an environment.[4, 152] Some molecular chaperones may also possess "unfoldase activity"[153] to permit misfolded proteins to escape from kinetic traps of the type discussed in Section 7. Their action can also be discussed in terms of energy surfaces such as those shown here for the folding of isolated systems.[154] However, at the present level of information, simple kinetic models yield satisfactory descriptions. Another intriguing problem is associated with the folding of proteins in membranes or at membrane surfaces. Experiments are just beginning to explore how this might occur.[155] Given the very large number of proteins associated with membranes, the nature of folding in a mainly hydrophobic, rather than hydrophilic, environment raises important questions.

In parallel with our developing knowledge of the principles of protein folding, it is being realized that folding and unfolding play an important role in the mechanism and control of a wide range of cellular processes. These include the translocation of proteins across membranes to their correct cellular compartments[155] and the regulation of the complex events occurring throughout the cell cycle.[156] In addition, the failure of proteins to fold correctly and efficiently is being associated with the malfunction of biological systems, and a substantial range of diseases is now known to be associated with the misfolding of proteins.[157] Some of these diseases, for example cystic fibrosis, result from mutations which interfere with the normal folding and secretion of specific proteins.[158] Others, such as Alzheimer's and Creutzfeldt – Jakob diseases, are associated with the later conversion of normal soluble proteins into insoluble amyloid plaques and fibrils.[159] Although such intermolecular interactions represent regions of conformational space outside those discussed in this review, the origin of the forces and the nature of the folding process is expected to be similar, except for the presence of the concentration dependence as an additional variable. There also exist normal proteins in which the monomer folds efficiently in dimeric or multimeric form.[160, 161] One of the proteins for which mutations are known to result in an amyloid-based disease is lysozyme,[162] whose folding behavior as a monomer was discussed extensively here. Recent studies have suggested that the aggregation-prone species giving rise to the formation of amyloid fibriles may be closely related to normal folding intermediates. A more detailed understanding of both normal and deviant folding behavior of proteins could therefore be important in efforts to develop novel therapeutic strategies to avoid or reverse the onset or consequences of such diseases.

We hope that we have been able to demonstrate that the mechanism of protein folding is one of the most fascinating problems in the field of chemical reactions. This review will be a success if it awakens the interest of chemists in this rapidly evolving subject and persuades some of them to contribute to its investigation.

Appendix 1. Lattice Models of Protein Folding

In a lattice model, the residues are positioned on a grid and have specific nearest neighbour interactions which depend on the residues involved. A cubic grid is used in the models discussed here,[28, 54a] although more complex lattices have been employed.[146] The folding process on the lattice takes place by local Monte Carlo moves of the residues until the native (lowest energy) configuration is reached. To illustrate the lattice models used in Figures 6 and 7, we consider a simple polypeptide chain with 27 amino acids (27-mer) and a folding trajectory of that chain. There are on the order of 10^{16} conformations of the chain on an infinite cubic lattice. Given a net hydrophobic attraction between the residues, the native state of the model protein corresponds to a $3 \times 3 \times 3$ cube (the structure on the far right in figure 13 d), which is fully occupied by the 27-mer polypeptide chain. There are 103 346 ways of threading a 27-mer through the cube. Which one of the possible threadings is the native state is determined by the sequence, that is, by the nature of the interactions between neighboring pairs of amino acids in any given conformation. Since there are 28 nearest neighbor contacts in the cube, the native state can be specified in terms of these contacts. In the model, simple contact potentials are used to represent the effective energy of a given configuration. This makes it possible to determine the thermodynamics and dynamics of the system with a reasonable amount of computer time.

Although the potentials used in the lattice simulations are simplified, the results provide general information on possible protein folding scenarios, which are the primary concern of this article. To determine the detailed behavior of specific proteins, more elaborate models and more accurate interaction potentials are required. The insights from folding simulations with lattice models and the complementary results of detailed all-atom model unfolding simulations can be combined with experimental data to achieve an understanding of protein folding. The contribution made by the simulations is that they enable the structures, energetics, and dynamics of reactions to be analyzed at a level of detail not accessible to experiment.

To illustrate the time course of a trajectory at a temperature near the folding transition temperature T_m, the energy E, the number of contacts N (a measure of the compactness that is similar to the radius of gyration), and the fraction Q of native contacts are shown in Figure 13 e. For a 27-mer, N goes from zero for the fully extended chain (no contacts) to 28 for the fully compact cube, whether or not it is in the native state. The number of contacts N increases before Q. This corresponds to a rapid collapse (in 10^4 Monte Carlo steps) and the formation of a disorganized relatively compact globule. The collapse is followed by a slow search (10^7 steps) through the 10^{10} semicompact states (the structure changes completely over about 10^3 steps, but the number of contacts remains high) until one of 10^3 states in the transition region is found. Since Q in the transition region is between 0.7 and 0.9 (which means 20 to 26 native contacts), the transition states resemble each other and the native state. From there, as expected for a transition state, there is rapid rearrangement (10^4 steps) to the native state.

An approximate relation between the number of Monte Carlo steps and the time can be obtained since the pseudodihedral angles in the lattice model (e.g., the dihedral angle formed by amino acids 2–5 in Figure 13 d) can be identified with the corresponding C_α-C_α-C_α-C_α pseudodihedral angles of a protein backbone. The rate for a change in the C_α pseudodihedral angle is on the order of $10^9\ s^{-1}$;[166, 167] therefore, 10^6 Monte Carlo steps correspond to about 10^{-3} s. In a typical folding simulation of the model protein, approximately 10^7 steps are required; this corresponds to 10 ms, a value comparable to that found experimentally for the folding of small proteins.

Appendix 2. Structural Studies of Protein Folding

Protein folding can be initiated in the laboratory in a variety of ways. One of the simplest is to unfold the protein in a high concentration of chemical denaturant, such as guanidinium chloride, and then to dilute the solution rapidly such that the denaturant concentration falls below that at which the native state is thermodynamically unstable. The question then is how to follow the structural changes that occur, given that folding is often complete in less than 1 s, and that (as the energy surfaces indicate) the structures of folding chains are extremely heterogeneous, at least until the final stages.

One strategy that has been developed is to use a battery of complementary stopped-flow and quenched-flow techniques, each of which is capable of monitoring a specific aspect of the formation of nativelike structure.[65, 98] This approach is illustrated schematically in Figure 14. For example, if the far-UV CD signal is followed as a function of time, the development of secondary structure (particularly helicity) can be monitored. If, in contrast, the near-UV CD signal is measured, the development of nativelike packing of aromatic side chains can be probed. The range of techniques being utilized in this way is increasing rapidly; one of the more remarkable developments is the ability to follow the overall dimensions of the protein (R_g) by means of time-resolved small-angle X-ray scattering.[69]

The dead time in even the best conventional mixing devices is typically a few milliseconds, but progress is being made to develop devices in which this is substantially reduced.[130] In addition, novel methods to initiate refolding reactions are being introduced.[138-142] These include the use of temperature jumps under conditions where cold denaturation takes place and an increase in temperature leads to refolding.[139, 141] In some cases, the rapid change of oxidation state of a metalloprotein can trigger the onset of the folding reaction.[140] With

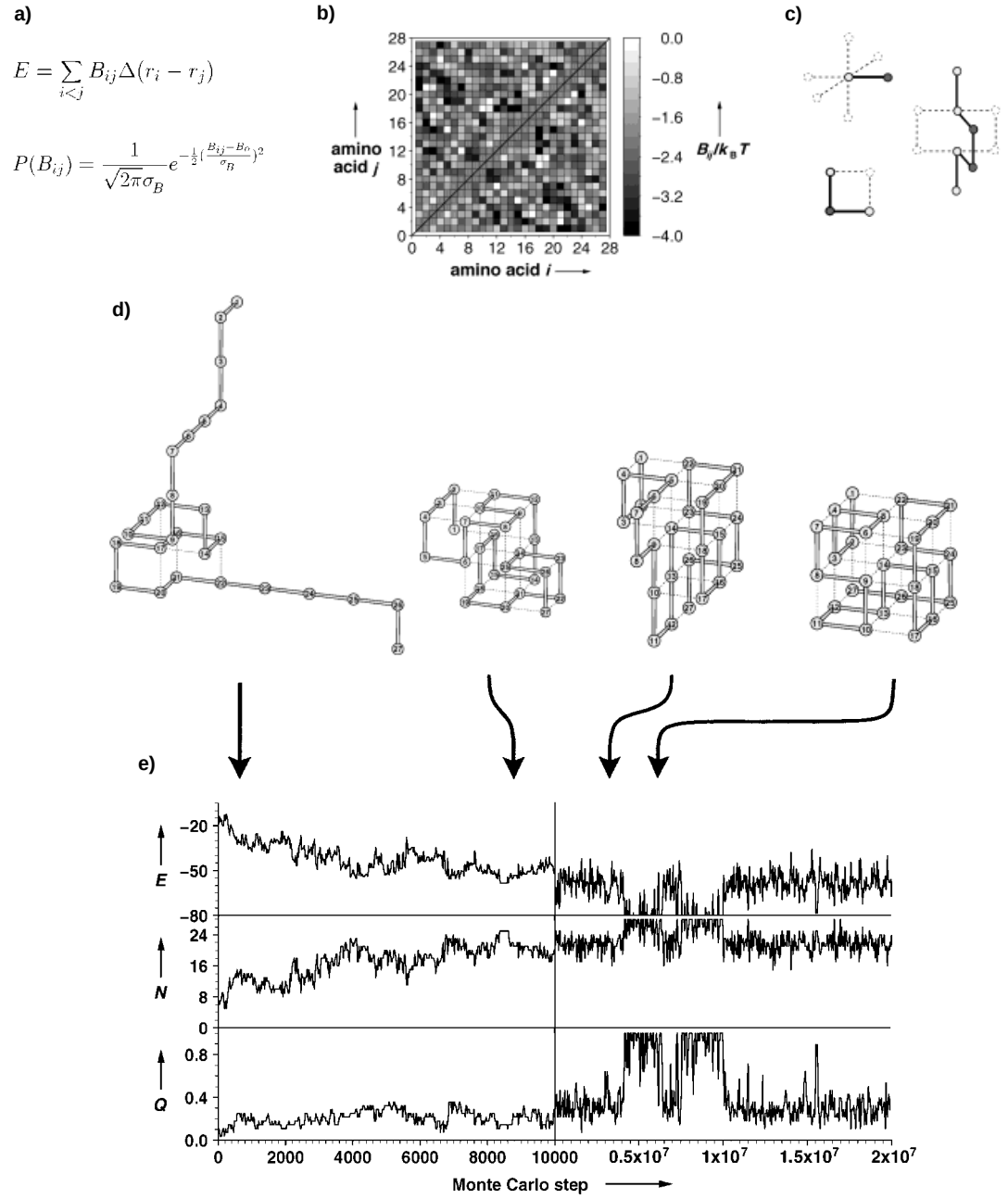


Figure 13. a) The total energy E of any chain configuration is a sum of residue contact energies B_{ij}, which themselves are derived from a Gaussian distribution with a mean value of B_0 and the standard deviation σ_B. The parameters r_i and r_j represent the position vectors of the residues with the sequence numbers i and j, respectively. The value of B_0 is $-2\,k_BT$ and corresponds to the average attraction between the monomers with which the hydrophobic effect is emulated. The standard deviation σ_B is k_BT and corresponds to the range in the contact energies of the amino acid residues. b) The contact energy matrix for the sequence used in e) and in Figure 3. c) The three allowed Monte Carlo moves: tail flip (top left), corner flip (bottom left), and the crankshaft move of two residues (right). d) Sample structures for the various parts of the folding trajectory shown in e), as indicated by the arrows (from left to right): a random coil, a disorganized semi-compact globule, a transition state, and the native state. f) Folding trajectory showing E, N, and Q as a function of the number of Monte Carlo steps; adapted from references [28, 55].

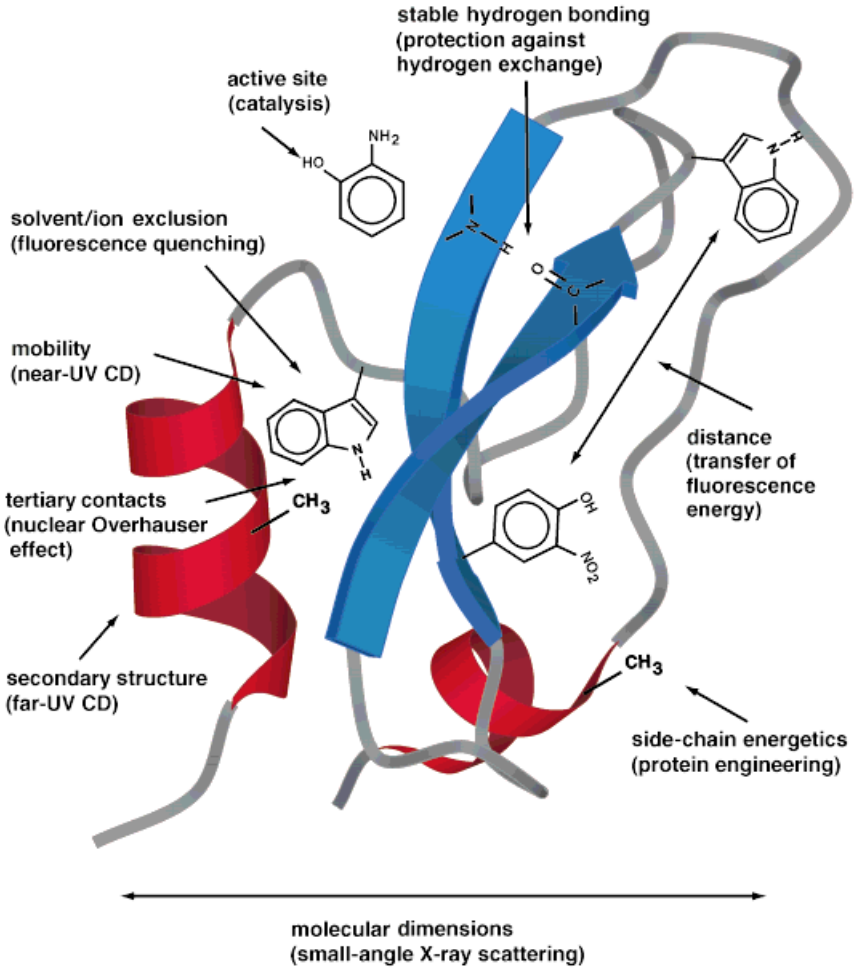


Figure 14. Schematic representation of some of the characteristics of a globular protein that can be followed during folding by physical techniques (stopped- or quenched-flow) with time resolution in the second to millisecond range; adapted from reference [130]. Examples of specific techniques able to probe the different features are indicated. Other properties that can be monitored but are not indicated include the existence of hydrophobic cavities and overall thermodynamic stability. Although no individual probe can monitor all of the structural changes during folding, the use of a set of complementary probes can provide a detailed picture of the distributions of conformations that are sampled at different stages of folding.

such approaches folding events on the micro- and submicrosecond time scale are becoming accessible.

A particular aim of structural studies is to probe interactions as they form at the level of individual residues. It is such data that can be compared directly with simulations. NMR spectroscopy has long been recognized as the approach by which this can be achieved, at least in principle. Considerable progress is now being made at realizing this goal in practice. If folding is relatively slow, on a time scale of seconds or longer, one-dimensional spectra can be recorded sequentially, and the spectral changes monitored.[131, 132] One example of such an experiment is depicted in Figure 15. Moreover, it has been shown that it is possible to monitor the folding reaction at the level of the individual residues of an isotopically labeled protein with a single two-dimensional experiment; this opens the way to high-resolution experiments.[83] Equally important has been the demonstration that laser-excitation methods of generating nuclear polarization (photo-CIDNP experiments; CIDNP = chemically induced dynamic nuclear polarization) allow the time scale on which events can be followed to be reduced to about 10 ms,[133] which brings time resolution of this NMR technique close to that of optical spectroscopic methods.

Another approach to using NMR spectroscopy to follow folding has been through hydrogen pulse labelling procedures.[50, 168, 169] This approach is based on the fact that labile hydrogens (e.g., those attached to the amide nitrogen atoms in peptide bonds) exchange rapidly with solvent hydrogen atoms in unfolded states, but are often protected from exchange when the protein folds as a result of the involvement of the amides in hydrogen bonds and through burial in the protein interior. At different times after initiation of the folding of a protein, the extent of protection of individual hydrogens can be monitored by applying at high pH a "labelling" pulse in which exposed amide hydrogens are replaced by deuterons. The exchange is then "quenched" at low pH (to slow hydrogen/deuterium exchange), and the protein is allowed to refold. The extent of labelling at different sites (monitored by NMR) then reflects the nature of the protein structure at the time of the labelling pulse during the folding process. The extent of labelling can also be monitored by mass spectrometry, an approach that provides key information on the distribution of structural heterogenicity during refolding.[170]

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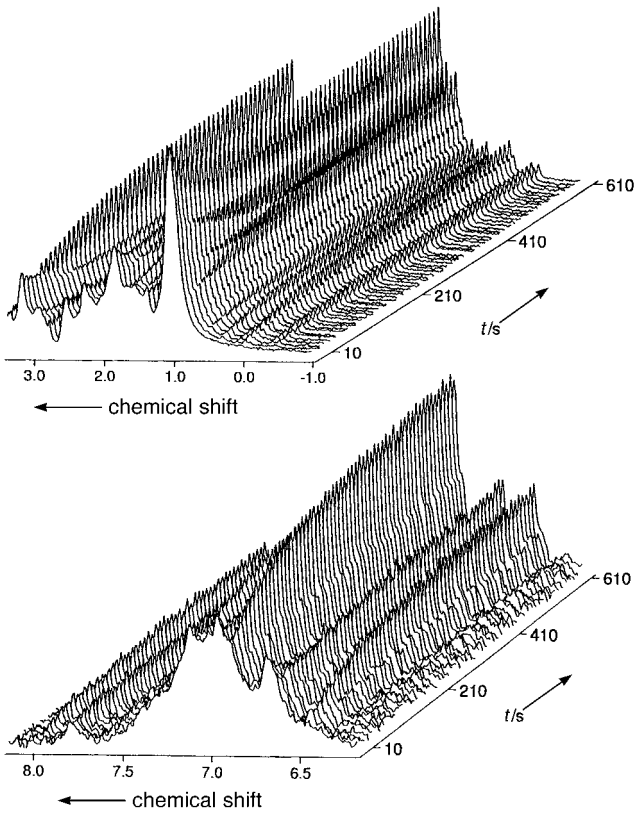

Figure 15. ^{1}H NMR spectroscopic investigation of protein folding in real time. Bovine α-lactalbumin dissolved in 6 M guanidinium chloride was injected into refolding buffer within an NMR instrument. Spectra were recorded at incremental time points between 1.2 s and 10.3 min after initiation of refolding; the stack plots show the changes in the spectral regions containing resonances for methyl and methylene groups (top) and aromatic side chains (bottom). The disappearance of the broad and overlapping signals of the initially formed disorganized globule state and the emergence of the well-resolved resonances characteristic of the native state are clearly evident; taken from reference [132].

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