BARRIERS IN PROTEIN FOLDING REACTIONS

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I. INTRODUCTION*

A. Overview

Protein folding reactions are distinguished from many other condensed phase reactions in several significant ways. First, the transformation of a random polypeptide chain into a unique three-dimensional structure involves breaking and forming many weak bonds, as opposed to one strong bond in classical chemical reactions. Second, hydrogen bonds between the protein and the solvent water, as well as hydrogen bonds between water molecules, are known to play a significant role in folding of the polypeptide. Third, the dramatic increase in the size of the conformational ensemble for a polypeptide undergoing an unfolding reaction and the well-known hydrophobic effect arising from the increased ordering of water solvating nonpolar amino acids buried in the native state and exposed when unfolded imply that entropy is a very important factor (1, 2). Thus, descriptions of folding reactions are complicated by a delicate balance between the enthalpy and entropy of the protein-water system. Finally, the barriers encountered by an ensemble of unfolded conformations as they proceed to the native state can be thought of as arising from the incomplete compensation between the changes in entropy and enthalpy of the system, rather than as high-energy, strained states. The nature of these barriers (i.e., their molecular, dynamic, and thermodynamic characteristics) remains a central focus of protein folding studies, with the expectation that understanding the rate-limiting processes is essential to answering the question of how proteins fold.

How barriers in protein folding reactions are viewed has evolved significantly over the last five years. These changes have been shaped by both experimental and computational studies. The discovery of proteins whose denatured ensemble folds to the native state either entirely or partially without the presence of intermediates (3) has focused increasing attention on the barriers or transition states of the folding reaction. These systems have also revealed that globular proteins can fold in unexpectedly fast time-scales, in some cases within tens of microseconds.

* Abbreviations: aTS, α-subunit of tryptophan synthase from E. coli; BPTI, bovine pancreatic trypsin inhibitor; CI2, chymotrypsin inhibitor 2; CspB, cold-shock protein from Bacillus subtilis; DHFR, dihydrofolate reductase; GCN4-p1, coiled-coil region derived from the yeast transcriptional activator GCN4; h, Planck constant; k_b, Boltzmann constant; m, sensitivity of equilibrium Gibbs free energy to denaturant concentration; m_f, sensitivity of Gibbs free energy of activation to denaturant concentration; MD, molecular dynamics; N, native state; NMR, nuclear magnetic resonance; PRA isomerase, N-(5'-phosphoribosyl)anthranilate isomerase from E. coli; T, temperature; U, unfolded state.
These findings led others to further explore whether intermediates in protein folding reactions are advantageous or simply traps that slowed folding, as suggested by lattice model simulations (6–8). Concurrently, triggered by the application of new technologies such as hydrogen-exchange nuclear magnetic resonance (NMR) (9–12), electrospray mass spectrometry (13, 14), temperature jumps (15–18), pressure jumps (19, 20), rapid mixing (21–23), and mutagenesis to the protein folding question, detailed characterizations of very early folding intermediates in more complex systems have been made. The results have yielded insights that complement studies on two-state* folding proteins. The role of intermediates in controlling folding reactions remains an open question and examples of obligatory and nonobligatory (7, 29–31) (i.e., productive and nonproductive) intermediates have been found.

Computational and theoretical studies have also had a major impact by bringing to the forefront the ensemble nature of the denatured, partially folded, and transition states of proteins, a point that was apparently insufficiently appreciated, or at least discussed, in earlier experimental work. These developments helped formulate the energy landscape view of protein folding (32, 33), which emphasizes the numerous, yet convergent, paths available to the unfolded state in reaching the native conformation and the description of the transition state ensemble as a collection of rare, high-energy conformations. Although in some lattice models there does not appear to be a structurally similar set of conformations defining the transition state ensemble for folding, recent molecular dynamics simulations and more protein-like lattice models (34, 35) indicate this may not be a general phenomenon. Thus, one of the major outstanding issues in protein folding concerns structural heterogeneity of these transition state ensembles and the role of this heterogeneity in determining folding pathways. These topics are currently being studied on both experimental and theoretical fronts, as will be discussed in this review and in other chapters of this volume.

B. Scope of this Review

The emphasis of this review will be on the experimental thermodynamic and structural characterization of the barriers in the folding of single-domain globular proteins. The first part of this chapter will focus

* In the course of this review we use the term *two-state* to indicate the absence of populated folding/unfolding kinetic intermediates. The presence of a slow minor amplitude phase for C12 (24, 25), α-spectrin (26), common-type acylphosphatase (27), and acylcoenzyme A (28) suggests that a formal kinetic description of the entire folding reaction would require consideration of additional states.
on the structural aspects of the transition state of folding reactions, primarily on mutational analyses and other protein engineering studies in which folding and/or unfolding kinetics have been investigated. The thermodynamic properties and the dynamics of the barrier crossings are addressed in a subsequent section. Studies of the elementary events in protein folding via simple model systems and a comparative overview of insights obtained from experimental and computational results complete the chapter.

Many single-domain proteins, typically composed of 300 or fewer amino acids, exhibit intermediates in their folding reactions. Studies in which the properties of the intermediates have been the focus, as opposed to the transition states, are covered in separate chapters of this volume. Reviews of independently folding subdomains, compact denatured states, and multi-subunit proteins are also addressed in other chapters of this volume.

II. FORMALISMS FOR THE QUALITATIVE AND QUANTITATIVE DESCRIPTION OF REACTIONS

A. Energy Landscape, Funnels, and the Reaction Coordinate

In describing protein folding reactions, pioneers of the field used terminology and nomenclature that were established in small-molecule chemistry (36). Concepts such as "reaction coordinate" and "transition state" have also played a vital role in the description of condensed phase reaction dynamics, such as electron transfer and solvation dynamics (37). Similar to protein folding reactions, many degrees of freedom exist in these systems. The reaction coordinate can be described as giving a reduced two-dimensional description of the progress of a reaction along a multidimensional potential energy surface where the properties of the remaining degrees of freedom are accounted for by introducing entropy and friction. However, when describing protein folding reactions, the proper choice of a reaction coordinate is ambiguous owing to the high dimensionality of the process. The multidimensional nature of the potential energy surface describing a protein folding reaction, although implicit in free energy reaction coordinate diagrams, was fully appreciated by the insights obtained from lattice models and energy landscape theory (32, 38). Folding funnels and other multidimensional representations of potential energy versus conformation have highlighted the roughness and traps in the energy surface and also attempt to convey the interplay
between entropy and enthalpy in folding to a unique native structure from a vast number of unfolded polypeptide chain conformations.

The approaches can be made complementary, however, in that the reaction coordinate can be viewed as the projection of the multidimensional potential energy versus conformation surface onto a particular order parameter such as the number of native contacts, radius of gyration, surface area, and other axes amenable to experimental study (39, 40). Both representations may be used to emphasize different aspects of the folding reaction. The barrier is frequently visualized, especially by experimentalists, who use the more traditional reaction coordinate diagrams, as free energy versus burial of solvent-accessible surface area. However, some workers have also employed funnels in interpreting their results (5, 41). A drawback of visualizing folding reactions via a reaction coordinate is that information on the structural details and opportunities for multiple pathways may be obscured. Additionally, the use of a single reaction coordinate to describe a folding reaction requires a concerted behavior of various order parameters (39, 40). Thus, the description of a folding reaction via a reaction coordinate diagram is enhanced by specification of the order parameter being monitored and by probing multiple order parameters.

B. Formalisms for Interpretation of Condensed Phase Reactions

Both elementary protein model systems and complex multistate protein folding reactions have, in many cases, shown an exponential or multieponential time dependence. Most workers have interpreted this to imply that a single barrier or several barriers are present along the reaction coordinate(s), rather than a distribution of barriers. The observation of the same kinetic time constants, within experimental error, using different experimental probes (42) and the presence of distinct structural features (43) have strengthened arguments for an exponential response in favor of more complicated phenomenologic descriptions (e.g., power law or stretched exponential). Although nonexponential kinetics have been observed in protein dynamics following ligand dissociation at low temperatures (44), there have been few such reports for folding reactions (45, 46). Probes of folding reactions on the nanosecond (47, 48) and microsecond (49) time-scales have also yielded exponential responses in protein folding, although some workers question the existence of a barrier in the dynamics of the initial chain collapse (50). Thus, reaction rate theory continues to be used in the analysis of experimental results and we briefly state below some of the strengths and shortcomings in its application to protein folding.
The general form of the rate expression given by Eyring and Kramers (37, 44, 51-53) is the product of a preexponential factor, $v$, and an exponential term describing the activated nature of the reaction:

$$k = ve^{-\beta E_a}$$

(1)

In protein folding reactions, $E_a$ has been associated with the activation free energy $\Delta G^\ddagger$ and $\beta$ with $1/RT$. Proper treatment of the preexponential factor, $v$, is a central question not only in protein folding (54) but in chemical physics in general (37). In the Eyring equation, the expression $v = \kappa (k_b T/h)$ is derived from a quantum mechanical treatment of the reaction coordinate. $\kappa$ is essentially an arbitrary factor to take into account recrossings of the barrier not explicitly included in the theory. A smooth reaction coordinate is obtained that neglects the influence of the environment (i.e., the other degrees of freedom) and suggests an inertial crossing of the barrier (Fig. 1A).

More appropriate for folding reactions and condensed phase dynamics is the formalism introduced by Kramers and extended by others (37, 53, 55) in which the role of Brownian motion or diffusive dynamics on the barrier crossings is a key factor (Fig. 1B). In this case the preexponential is, in the overdamped regime expected for protein folding reactions (44), given by $v = \kappa (\omega_n/\gamma) (\omega_n/2\pi)$, where $\omega_n$ is the undamped angular frequency of the minimum of the potential well, $\omega_n$ is the frequency of the transition state, and $\gamma$ is the friction term. Friction arises from the coupling of vibrational and bath modes to the reaction coordinate and introduces the possibility of multiple recrossings of the barrier. A consequence of this exchange of energy and momentum with other degrees of freedom is that the time-correlation function of a particular vibrational, torsional, or displacement motion responsible for a reaction may be strongly affected. Damping of this motion by coupling to other modes of the protein or the solvent may significantly reduce the observed reaction rate compared to that predicted by transition state theory. Kramers' theory has yielded good agreement between theory and experiment for small molecules under a wide range of conditions, correctly describing the nonmonotonic dependence of rate on friction from low-pressure gases ($k \propto \gamma$) to viscous condensed phase reactions ($k \propto 1/\gamma$) (37, 53) (Fig. 2).

Application of this formalism to folding reactions, however, poses challenges in that it requires examination of the relevant protein modes and fluctuations participating in folding reactions (56-58). Additionally, significant frictional effects on protein dynamics have been attributed
A schematic illustration of an inertial barrier crossing (A) and a diffusive barrier crossing (B). In the inertial barrier crossing assumed by transition state theory, once the barrier is traversed, the particle is trapped in one of the quasistable states. Many recrossings, however, may occur in the diffusive barrier crossing before the product state is obtained. The Brownian dynamics of the barrier crossing in the lower panel are a manifestation of friction along the reaction coordinate in Kramers' formalism [adapted from Fleming and Wolynes (37)]. The smooth reaction surface shown is a simplification of what is most likely, for protein folding, a reaction coordinate with ruggedness superimposed on it.

to the solvent (59-61) and also to the protein matrix itself (62), the latter possibly originating from the dense packing of residues in the interior of proteins (61) (Fig. 2). The diffusive nature of barrier dynamics and the appropriateness of Eq. (1) in protein folding reactions have been supported by simulations of lattice models (8) and in studies of torsional dynamics of aromatic residues (60). The effects of friction on
Friction

Fig. 2. Qualitative description of the dependence of the reaction rate on the (external) friction. Because transition state theory (TST) does not incorporate a friction term, the reaction rate is independent of friction. The dependence of the reaction rate for small molecules on friction in Kramers’ model is shown by the solid line (37). In the modified Kramers’ expression (dotted line) (62), the relatively constant internal friction of the protein molecule can become rate determining as the external friction (e.g., viscosity) is reduced.

folding dynamics can be probed experimentally by external perturbations such as viscosogens, pressure, and temperature without a priori knowledge of the absolute magnitude of the pre-exponential term (37, 63).

With these caveats in mind, quantifying the barriers of protein folding reactions has relied primarily on the transition state formalism [Eq (1)]. With the accessibility of faster experimental time-scales of the folding reaction, the value of the pre-exponential term has received renewed interest as an indicator of a “speed limit” for a barrierless protein folding reaction (i.e., ΔG‡ = 0) (64). Eaton and coworkers have suggested an upper limit of 10⁶ s⁻¹ for this value based on studies of intramolecular ligand binding in cytochrome c (54), while other studies have suggested this value to be closer to 10¹⁰ s⁻¹ (33, 41, 61). The dynamics of carbonmonoxy hemoglobin following ligand photo-disassociation have been satisfactorily described using a value closer to 10¹³ s⁻¹ (44). Whether a single value for this parameter is appropriate for different types of rate-limiting steps, as in nucleation-condensation versus diffusion-collision (discussed below), is not clear. One can, for example, envision fluctuations about a metastable minimum dominating the rate for a nativelike
collapsed structure whereas diffusion of the polypeptide chain may be significant for early folding events.

III. AN OVERVIEW OF PHENOMENOLOGICAL MODELS OF PROTEIN FOLDING

A. Nucleation-Condensation

In protein folding reactions governed by nucleation-condensation reactions or nucleation-growth reactions (65–69), the secondary structural elements or fragments of the protein are considered to have poor stability by themselves and do not appreciably populate nativelike conformations. Stabilization of these elements usually requires interactions involving nonlocal residues, typically in a structural unit, or foldon. Because approximately 70–100 amino acids are thought to be necessary for an autonomous structural unit (70) (see also the chapter by Peng and Wu in this volume), large single-domain proteins may contain more than a single folding nucleus. Theoretical estimates of the average size of the folding nucleus are approximately 10–15 residues (71). Formation of secondary structure and native-like tertiary contacts occur in a concerted fashion in the transition state with several key residues forming the most favorable contacts. Events following this rate-limiting step are not observed or probed because of the rapidity of subsequent step(s) leading to the final native structure. This folding model is distinguished (65) from classical nucleation by the weak bias toward strong local interactions. Therefore, structure does not develop into a large organized unit from only a few residues with local structure. These ideas concurrently grew out of experimental mutational analysis (72, 73) and lattice models (66, 68, 71). A recent review on nucleation-condensation mechanisms has been provided by Fersht (65).

B. Diffusion-Collision

The diffusion-collision model reduces the folding process into a sequential coalescence of microdomains, structural units (e.g., helices, β-hairpins) small enough to rapidly search their entire conformational space (74, 75). Because the rate of microdomain coalescence increases as larger, more stable, macrodomains are formed, the folding process is cooperative (60). This hierarchic process (76, 77) is distinguished from the nucleation-condensation model by a stronger bias toward the formation of secondary structural units in the transition state, which may precede the formation of native-like tertiary contacts distant in
sequence. However, a nucleation-collapse scenario is not excluded because of the generality of the theory and flexibility in the parameterization. The folding rate is determined, to a significant extent, by the local stability of isolated microdomains and their topology-dependent probability of successful collision via diffusion. Whether folding proceeds by a single pathway or a multitude of pathways is dependent on the properties of the microdomains, which in turn depend on the amino acid sequence. An attractive feature in the formulation of the diffusion-collision model is that it lends itself to relatively straightforward quantitative comparisons between experiment and theory as discussed in Section VII,B.

C. Hydrophobic Collapse

The central characteristic of the hydrophobic collapse model is the tendency of hydrophobic residues to be excluded from the aqueous environment. Early events in folding lead to a collapsed ensemble of structures in which the conformational search space has been drastically reduced by the clustering of hydrophobic residues in the interior of the nonspecifically collapsed species (2, 38). The search for the native conformation proceeds on a longer but still feasible time-scale from this reduced set of conformations that exhibit relatively fluid structures and have been referred to as molten globules (78–81). The collapse promotes the partial formation of secondary structure but not specific tertiary contacts. An alternative view is that a bias toward native contacts is present but non-native contacts may also be formed, as envisaged in a funnel-like landscape (40, 82–84). This scenario is distinguished from the nucleation-collapse model, where the nucleus is considered an activated state, in that the collapse is nearly activationless. Therefore, the collapse is kinetically favored, and its formation is significantly more rapid under conditions favoring the native state (50).

As data on an increasing number of various structural types of proteins have accumulated, it is becoming recognized that these phenomenological models are not all encompassing but rather represent the dominant characteristics of specific limiting cases of folding reactions. This point is illustrated by recent cubic lattice models in which the balance between native and non-native attractive interactions can change the folding from one driven by a hierarchic pathway as in diffusion-collision to one controlled by a delocalized folding nucleus as in nucleation-condensation (83). A similar effect of the interplay between local versus nonlocal interactions on folding mechanisms was highlighted in an extension of Zwanzig’s analytical folding model (85, 86). Reinforcing
these points, it has been suggested that for an optimal, barrier-free folding rate, secondary structure formation (governed predominantly by local interactions) and chain-collapse (dictated by nonlocal interactions) occur concomitantly (71).

IV. STRUCTURAL FEATURES OF BARRIERS IN PROTEIN FOLDING

A. Secondary Structure

A significant aspect of studies on small synthetic peptides and those isolated from naturally occurring proteins was the unexpected tendency of these structures to adopt stable helical conformations in isolation (87–89). These studies led to the hypothesis that preformed helical structures played a key role in limiting the conformational search space in protein folding. Mutational analysis studies and refinements in helix-coil theory (89–94) have been essential in testing this hypothesis.

One of the simplest systems in which a systematic study of the secondary structure content in the transition state was carried out is the dimeric coiled-coil GCN4-pl (95–98). The two-state refolding kinetics of this 33-residue peptide exhibit a bimolecular rate constant several orders of magnitude less than that expected for a diffusion-controlled reaction in solution. The slow rate was attributed to the marginal population of association-competent helical monomers in rapid equilibrium with unfolded monomers (97). A subsequent study employing alanine substitutions in the exterior f-positions of the heptad repeat unit to vary the stability and population of helical monomers, however, suggested that the refolding rates were not correlated with helix propensity. This result was interpreted to mean that the dimeric transition state contained little helical structure (98). More recent data (J. Zitzewitz, personal communication) employing simultaneous substitutions at multiple f-positions, however, suggest that significant differences in rate are observed for substitution in the two heptads in the C-terminus but not the N-terminus. Consistent with this finding is that, unlike the N-terminal half of the peptide, residues 1–17, the monomeric C-terminal half, residues 17–33, has considerable helical structure (J. Zitzewitz, personal communication; see also Ref. 99). These results are also consistent with calculations of helical content using the secondary structure prediction program AGADIR (92) (see also the chapter by Serrano in this volume). The dimeric transition state for GCN4-pl may be composed of a relatively structured, helical C-terminal region and a much less-structured N-terminal region. Supporting this hypothesis are the results of a ki-
namic study of the infrared amide I region using nanosecond-resolved temperature-jump relaxation near the midpoint of the temperature-induced unfolding transition. A rapid, concentration-independent zipping/unzipping phase with a negative activation enthalpy was followed by the dissociation/bimolecular-association step (100). The transition state of GCN4-p1 may be viewed as having features consistent with that of a diffusion-collision model (preformed helical structure in the C-terminal half) and also with a nucleation-condensation model (rapid condensation of the N-terminal half following formation of the transition state), where the docked pair of C-terminal regions serves as the folding nucleus.

A significant role for helical structure in the transition state was also suggested by the results of alanine replacement experiments for surface positions in several helices of monomeric λ repressor (5). Although alanine substitutions also had a pronounced effect on the location of the transition state along the reaction coordinate in some cases (see Section VII,B), the importance of helical structure was not diminished. The presence of a nearly native-like α-helix was inferred from mutational analysis of the transition state of CI2 (72).

Mutational analysis of the two-state bimolecular association reaction of P22 Arc repressor led to the conclusion that association and folding occur concomitantly in the transition state (101). The intertwined monomers were suggested to disfavor significant preformed monomeric structure. However, the most significant refolding rate enhancement (k_{mut}/k_d \sim 2) occurs for the replacement of Ser-35 with Ala on the surface of a helix and the unfolding rate is unchanged. The greater helix propensity of alanine versus serine (90, 92) is expected to shift the unfolded ensemble to favor helix formation in this region and, thereby, accelerate folding. The proposed absence of preformed structure in the monomer also contrasts with that for a stable monomeric form of the all-helical trp repressor. Despite having similarly intertwined helices in the fully folded dimeric form, the monomer exhibits a non-native but well-folded structure (102).

Assessment of β-sheet/strand formation in the transition state using this approach is complicated by a significant role for nonlocal interactions in determining β-strand propensities (103, 104).

B. Tertiary Structure

Mutational analysis has been the primary tool for probing the presence of nativelike tertiary interactions in the transition state. The effect of mutations on the refolding and unfolding kinetics permits inferences
to be made regarding the environment of the side chains in the transition state. The effect of a mutation on the transition state free energy is obtained via transition state theory and normalized relative to its effect on the native state stability to obtain the $\phi$-value, $\phi = \Delta \Delta G_{\text{U}} / \Delta \Delta G_{\text{N\text{U}}}$. Advances in molecular biology have made it possible to test a great majority of the residues in several small proteins in this manner (72, 101, 105). A $\phi$-value of unity suggests that the side chain of the residue is in a nativelike environment in the transition state whereas a value of zero implies an environment that is unfolded-like. Intermediate values and values outside this range are observed and usually taken to indicate that only partially nativelike interactions are present or that native-like interactions are present a fraction of the time in the transition state. Although fractional $\phi$-values may be influenced by several competing and compensating factors, correspondence with $\phi$-values obtained from molecular dynamics simulations (see Section VII,A) have given support to the experimental values. The point mutations are usually assumed not to affect the folding mechanism, the structure of the unfolded state, and the structure of the transition state ensemble. However, drastic changes in the properties of the transition state can occur (4, 106). A study addressing more subtle effects of point mutations and denaturants on the properties of the transition state ensemble has also appeared (107). The preexponential factor in Eq. (1) is also assumed to remain invariant upon point mutation, unlike the case for loop insertions or extensions (see Section IV,D).

Studies of the effect of point mutations on refolding and unfolding kinetics have been performed for P22 Arc repressor (101), src (105) and $\alpha$-spectrin (108) SH3 domains, CI2 (72), and barnase (109). The insights obtained with mutational analysis are exemplified by the study on P22 Arc repressor, in which amino acid substitutions were made at 44 of the 53 residues. A linear relationship between $\Delta \Delta G_{\text{U}}$ and $\Delta \Delta G_{\text{N\text{U}}}$ for Arc repressor (and also for CI2), demonstrated that the energetic perturbation of the transition state is proportional to that of the native state for all of the residues investigated. This suggests that the transition state for these proteins, similar to other small proteins such as CspB (110, 111), is analogous to an expanded form of the native structure. The central hydrophobic core is partially formed even though it does not exhibit nativelike side chain packing density or specific hydrogen bonds.

In contrast to the relatively uniform distribution of $\phi$-values for several small proteins, the distribution for SH3 domains is more heterogeneous. Regions of native-like interactions in the transition state (e.g., the distal loop hairpin) are juxtaposed against relatively unstructured sections (RT loop, n-src loop) suggesting that the transition state is "structurally
polarized" (105). Additionally, the presence of a hydrogen bond network is suggested. The most structured regions also correspond roughly with those exhibiting the greatest protection from HD exchange with the solvent (105).

The heterogeneity exhibited by the SH3 domains and also by barnase (112) has been attributed to differences in topology between helical proteins and β-sheet proteins. Helical proteins have been suggested to have a more "delocalized nucleus" than β-sheet proteins (113). Additionally, illustrative examples of the significant effect of a point mutation on the folding mechanism and position of the transition state along a solvent accessibility reaction coordinate can be found in the helical proteins Arc repressor (114) and monomeric λ repressor (4).

The early folding intermediates of the single structural domain proteins dihydrofolate reductase (DHFR) and α-subunit of tryptophan synthase (αTS) exhibit a similar structural polarization (115–117). For DHFR, hydrogen exchange pulse-labeling (115), rotational correlation time measurements via time-resolved anisotropy (116), mutational analysis (228), and circular dichroism of excitonically coupled tryptophans (118) suggest that the central β-sheet is well formed whereas the adenine binding domain is relatively unstructured in the early stages of folding. Although αTS exhibits a similar structural heterogeneity early in the folding process (117), mutational analysis suggests the rate-limiting transition state for unfolding and refolding is more analogous to an expanded form of the native state (119, 120).

The src and α-spectrin SH3 domains have very similar native structures and ϕ-value distributions in the transition state despite only a 34% sequence identity. The conservation of transition state structure concomitant with sequence divergence has led Baker and coworkers (105) to suggest that topology rather than specific amino acid content dictates transition state structure. This argument is supported by the effect of circular permutants (see below) on the transition state structure as deduced from ϕ-value analysis. The generality of this observation, however, is yet to be established. In a study of fatty acid binding proteins, also predominantly β-sheet proteins, Ropson and coworkers found significant differences in their folding mechanisms (and presumably transition states) even though they exhibit the same fold and highly similar native structures (121).

Protein engineering studies on Arc repressor (114) and Rop (122) have demonstrated the presence of specific tertiary interactions in the transition state and their influence on its free energy. Replacement of a buried salt bridge and hydrogen bond interactions with a hydrophobic cluster was found to considerably lower the transition state free energy,
resulting in a 40-fold rate enhancement (114). Analogous findings for repacked cores of Rop (122) suggest that interactions requiring specific alignment may be more difficult and energetically costly to achieve in the transition state structure and may also be dependent on the ability of participating amino acids to search side chain rotamer conformations. Thus, tertiary interactions as well as topology (see Section IV,C) may be important in assessing the determinants of folding rates.

C. Effect of Topology

1. Contact Order

Baker and coworkers (123) have systematically tested the statistical correlations of the folding rate of 12 two-state small single-domain proteins against a number of parameters such as stability, size, chain length, transition state placement, and topology. They found the relative contact order, a parameter gauging the degree of local versus nonlocal contacts, to be the most statistically significant. The relative contact order is defined as the average sequence distance between all pairwise contacts normalized by the number of residues. Their analysis suggests that proteins with a lower contact order (i.e., greater influence of local interactions in sequence over nonlocal interactions) exhibit an increased folding rate. The underlying physics for the empirical relationship is not clear. Baker and coworkers (123) have noted contrasting views in which stabilization by predominantly local interactions would give rise to the opposite effect owing to increased roughness of the energy surface, stabilization of the unfolded form, and reduced uniqueness of the native state (see Ref. 71 for a review). If the correlation is valid, it reflects the significant narrowing of the conformational search space, and consequently a reduction in the entropic contribution to the barrier, early in the folding reaction, analogous to the diffusion-collision model. These observations have been corroborated in a recent refolding molecular dynamics simulation (124).

Although the contact order successfully captures some of the determinants to the folding free energy barrier for several small proteins, exceptions may arise when a delicate balance in stability between different local structural units is present. For example, β-lactoglobulin is known to fold through a highly helical intermediate that converts to the predominantly β-sheet native conformation in the rate-limiting step in folding (125, 126). Secondary structure propensity scales suggest that the sequences would prefer to be helical, consistent with the destabilization of the native form in acid, which results in a helical, molten globule–like
form (127). In this case, off-pathway intermediates or "traps" might actually slow down the folding reaction. Additionally, the sizable variation in refolding rates within structurally similar proteins and upon point mutations suggests that factors other than topology are also likely to be significant (3).

2. Circular Permutations

Rearrangement of the order of secondary structural elements by circular permutation (128) has been shown to lead to similar native structures, often with diminished stability, for a number of proteins: BPTI (128), ribonuclease T1 (129), α-spectrin SH3 domain (130), DHFR (131), CI2 (73), PRA isomerase (132), and T4 lysozyme (133). Kinetic folding studies of permuted proteins enable a test of the relative importance of local and nonlocal interactions in determining the stabilizing interactions in the transition state ensemble and in the early events of folding. By joining the N and C termini of the polypeptide chain with a peptide segment and introducing termini in another region, typically a loop, the interaction of particular secondary structural elements or residues and the role of the cleaved loop in the transition state and early folding steps may be probed.

A systematic study of the effects of permutation was carried out by Viguera et al. on the all-β, kinetically two-state protein α-spectrin SH3 (130). Refolding and unfolding kinetic studies on permutants in which termini were placed in each of the three loops were studied. Although the permutants were slightly less stable than wild-type and pseudo-wild-type versions, the refolding rates were significantly different. For one of the permutants, formed by cutting at a disordered loop and introducing a β-hairpin between the N and C termini, the substantially faster refolding rate was attributed to a change in the structure of the transition state. The linking of two β-strands, which normally attain native-like structure late in the folding reaction, presumably resulted in a change in the folding nucleus and, consequently, the folding pathway. Consistent with these results, subsequent thermodynamic analysis (see Section V,A) of the transition state revealed an entropic stabilization for the refolding barrier and an enthalpic stabilization for unfolding (134).

An analogous study was attempted on CI2 (73), but it was found that cleavage at only one particular site resulted in a stable protein. A mutational analysis of this permutant suggested a transition state very similar to the wild-type protein, indicating that the folding nucleus had been preserved. Differences between CI2 and α-spectrin have been proposed to arise from reduced permissiveness toward disruption of the folding nucleus in CI2 and the presence of multiple folding nuclei in
α-spectrin (73). Curiously, a consensus on the extent of localization of the folding nucleus based on computational lattice models has been lacking (66–69).

3. Circularization

Although circularized forms of several proteins have been reported (73, 135, 136), relatively few studies of their kinetics have appeared. For one study of circularized CI2 (73), formed by placing cysteines near the termini of CI2 and forming a disulfide link, Otzen and Fersht observed a marginal increase (~1 kcal⋅mol⁻¹) in native state stability and an approximately fivefold (12.8 versus 2.5 s⁻¹) increase in the refolding rate of the oxidized versus reduced species. Mutational analysis suggested a similar transition state for both species. The increase in folding rate for the circularized protein was therefore attributed to the reduction in conformational search space. The influence of the increased thermodynamic driving force, which has been shown to correlate with the refolding rate in some proteins (137) (see Section V,A), was not addressed.

D. Loop Lengths

An inverse correlation of loop length with native state stability has been shown to be consistent with the entropy of loop closure expected from polymer theory (138). The effect of variation of the loop length on the structure, energy, and dynamics of the transition state is less well established. The effective length of loops in the structure of transition states and their influence on the rate of folding reactions were tested in studies on CI2 (139) and the α-spectrin SH3 domain (26). In the study on CI2, insertions of various side chains and lengths (7 to 13 residues) were made into the existing loop of CI2 and shown to only marginally affect the stability and refolding rates. The slight retardation of the refolding reaction was reconciled with predictions from polymer theory assuming the dominant contribution to changes in the free energy barrier arises from changes in configurational entropy. In an analogous study in which insertions of 2 to 10 glycines were made in two circular permutants of α-spectrin SH3 domain, Viguera and Serrano (26) suggested that a contribution in addition to configurational entropy is responsible for the changes in rates. This conclusion was based on the inconsistency between the effective loop lengths of the transition state calculated from the unfolding and refolding data. The discrepancy was attributed to a significant contribution from intramolecular diffusion in forming the folding nucleus. This would suggest that elongation of the loops contributes more to the dynamics of the barrier crossing [i.e., the
preexponential term in Eq. (1)] than to the thermodynamics [i.e., the exponential term in Eq. (1)] of the transition state ensemble.

**E. Cooperativity: Fragment Complementation**

Pioneering work by Richards (140) on ribonuclease A showed that reconstituting a pair of proteolytically derived fragments can lead to a native-like noncovalent complex that overcomes the entropic stabilization of the dissociated state. Examination of the refolding complementation kinetics of protein fragments has more recently been used as a tool for drastically reducing the cooperativity of the folding of single chain proteins. The high cooperativity of many folding reactions, especially small two-state proteins, makes it difficult to identify the determinants of the stability of transition states. In dividing the chain into two independent segments, the search for favorable stabilizing contacts is biased heavily toward local contacts. The sampling of nonlocal contacts between the two segments is determined by their frequency of collisions, which is concentration dependent and typically orders of magnitude lower than if they were linked as a single chain. The second-order kinetics therefore permit a test of the role of preexisting structure and assess the relative kinetic and thermodynamic significance of local versus nonlocal interactions in determining the folding transition state for the full-length protein.

In studies on Cl2, Fersht and coworkers have shown that the isolated fragments are unfolded-like and their complementation results in formation of the native structure, as determined by NMR and X-ray. The association rate is approximately six orders of magnitude slower $(3.7 \times 10^3 \text{ M}^{-1} \text{s}^{-1})$ than a diffusion-controlled second-order rate constant $(141, 142)$. However, some non-native hydrophobic clustering in the fragments was observed and rearrangement of these local structures may contribute to the diminished rate. Unfolded fragments and comparable slow association rates $(1.3 \times 10^3 \text{ M}^{-1} \text{s}^{-1})$ were also found for fragments of thioredoxin (143) for which no residual structure was observed.

Mutational analysis has been shown to be a useful tool in identifying structural features of the transition state in the complementation kinetics. A correlation between the second-order complementation reaction and the two-state folding reaction of full-length Cl2 was established by a comparative mutational analysis of both reactions (141). Although the residual hydrophobic clustering in the fragments resulted in slightly different sensitivity to some mutations, the general observations were taken as evidence for the concerted formation of secondary and tertiary
interactions in the folding transition state for both the complemented and full-length proteins.

The role of small amounts of nativelike structure in relatively unfolded fragments was illustrated for barstar (144). One barstar fragment contained small amounts of helical structure similar to that found in the native structure and in the refolding transition state. The isolated complementary fragment was capable of inhibiting barnase. The association rate constant ($\sim 1 \times 10^5 \text{ M}^{-1} \text{s}^{-1}$) was nearly two orders of magnitude greater than that observed for the complementation reactions of the relatively unstructured fragments of Cl2 and thioredoxin but still several orders of magnitude slower than a diffusion-limited reaction. As in some dimeric proteins (97, 98, 145), successful association appears to require minute equilibrium populations of nativelike conformations. These flickering native-like local structures are only stabilized by the development of tertiary interactions in the transition state (142). The increased association rate constant with a greater extent of native-like structure is consistent with a diffusion-collision mechanism with a greater value of $\beta$, the parameter corresponding to the fraction of association-competent microdomains [see Section VII,B and Eq. (3)]. Fragment studies have been suggested as a means to obtain estimates of the stability of various microdomains in the parameterization of diffusion-collision type calculations (75).

The observation of native-like structure in protein fragments points to the importance of autonomous folding units in larger proteins (146, 147) (see also the chapter by Peng and Wu in this volume). Dissection or fragmentation of a protein may also be viewed as a significant and specific perturbation in topology. The extreme separation in time-scale between local and nonlocal collisions is reflected in the significant slowing down of the folding rate compared to the single-chain protein. However, the recovery of the native structure, as determined by NMR and enzyme activity, demonstrates that topology may selectively affect the barrier height relative to the stability of the native structure.

V. ENERGETICS AND DYNAMICS OF BARRIERS IN PROTEIN FOLDING

A. Dependence on Thermodynamic Driving Force

The presumption that the folding rate is dependent on the global thermodynamic driving force (i.e., the stability of the native protein relative to the unfolded state) is based in part on the expectation that certain residues in the transition state ensemble have native-like contacts.
If correct, then perturbations (e.g., mutations) altering the stability of the native state may also preferentially destabilize the transition state ensemble relative to the unfolded state. Mutational analysis can thus serve to address two related questions: (1) Which residues are involved in determining the height of the free energy barrier? and (2) Is the height of this barrier related to the overall stability of the native state or the stability of smaller structural units (e.g., the folding nucleus, microdomains, or secondary structural elements)? Mutational analysis also raises a related question: Are the folding pathways or, alternatively the contacts and structural elements in the transition state ensemble, conserved upon mutation of residues involved in stabilizing the transition state ensemble?

The dependence of the folding reaction and consequently the height of the free energy barrier on thermodynamic driving force has been addressed by several groups. Lattice models and analytical folding models have proposed that fast-folding proteins require a large thermodynamic driving force. Interestingly, alternative models, such as the specific-nucleus model (68) and simulations by Crippen and Ohkubo (35) do not exhibit this dependence.

An experimental test of these hypotheses was presented by Schmid and coworkers (110) in a study on the all-β-sheet protein CspB from three different strains in which the stability changed by ~4 kcal·mol⁻¹. For all three proteins, the two-state folding mechanism was preserved yet the refolding rate changed by only a factor of ~2. Apparently, the critical determinants to the folding rate and refolding barrier height are not necessarily dependent on the overall stability of the native state. The primary effect was observed as a change in the unfolding rate. Comparison of the nonconserved residues suggested that the dominant contribution to the stability differences was from surface residues that were not in native-like environments in the transition state ensemble. Most of the residues in the hydrophobic core of all three proteins were conserved, suggesting that the formation of a similar critical core of residues into a native-like packing serves as a rate-limiting event.

A complementary study by Plaxco et al. (137) on homologous human fibronectin domains with very similar core structures and identical topologies reached a strikingly different conclusion. In this study, also on an all-β protein, the species with a fivefold greater stability (6.1 versus 1.2 kcal·mol⁻¹) was shown to fold approximately two orders of magnitude faster. However, when the two proteins were studied at comparable stabilities very similar rates were obtained. These observations suggest that the determinants of the barrier height closely parallel those of the native state stability. Similar conclusions have been reached by other...
workers (148). The lack of a correlation between the activation free energy and the native state stability for CspB and also SH3 domains (149) may be reconciled by the presence of a local minimum between the activated state and the native state along the folding reaction coordinate, which can mask the effect of the stability change. Perhaps no corresponding minimum exists for the fibronectin domains.

Although there does not appear to be a general dependence on the stability of the native structure, a correlation of the rate of folding on the stability of local structural units has been demonstrated in the folding of monomeric λ repressor (5). The diffusion-collision model predicts that the rate of folding is dependent on the probability of a successful collision between two microdomains or secondary structural units. Oas and coworkers (5) systematically placed Gly → Ala mutations at surface residues in this α-helical protein in an effort to increase the stability of the isolated helices, but not necessarily the native protein. The observed folding rate was compared with that predicted by the diffusion-collision model and helix/coil theory. Their study is significant in two respects. First, that stabilization of local native secondary structural elements also results in stabilization of the transition state ensemble further points toward the role of preformed secondary structural elements in the transition state. Second, stabilization or destabilization of these local structural elements may result in significant changes in the dominant folding pathways and rate-limiting events (4). Alternatively, this phenomenon may be qualitatively viewed as a drastic change in the placement of the transition state, consistent with the large change in transition state solvent accessibility, measured by $\alpha = m_1^i/(m_1^j - m_0^j) = m_1^i/m_\text{eq}$. The $m^i$-value reflects the sensitivity of the rate, $k$, to denaturant concentration, [$D$], via $k = k_0 \exp(-m^i [D]/RT)$. The parameter $\alpha = m_1^i/(m_1^j - m_0^j)$ serves as a measure of the change in solvent-accessible surface area between the transition state and the stable states. A value close to unity corresponds to a refolding reaction in which the transition state is similar to the product state and a value near zero would suggest a reactantlike transition state. This alternative interpretation is shown schematically in Fig. 3, where the highest barrier occurs early along the reaction coordinate and is possibly associated with the docking of helix 3. The alanine mutations (G46A/G48A) preferentially stabilize this barrier relative to the later barrier along the reaction coordinate, resulting in a more native-like transition state and an accelerated folding reaction.

As described above, the role of preformed secondary structural elements was also addressed in the coiled-coil dimer, GCN4-p1. Although an early study, incorporating only single f-position replacements (98), concluded that the bimolecular association rate constant does not corre-
FIG. 3. Schematic representation via a reaction coordinate diagram of the proposed effect of the G46A/G48A mutation of monomeric λ repressor [see Burton et al. (4, 5)]. For the wild-type protein, the kinetic $m^1$-values from NMR linewidth measurements suggest the transition state (TS1) is closer to the unfolded state (U) on a solvent accessibility reaction coordinate (solid line). The G46A/G48A mutation stabilizing helix 3 lowers the free energy of TS1, resulting in a significant apparent shift in the location of the transition state (TS2) toward the native state (N).

late with the predicted stability of the independent helices, after accounting for side-chain interactions, this view has been challenged. Myers and Oas (229) have reanalyzed the results of Sosnick et al. (98) using a diffusion-collision formalism. They showed that, consistent with the mechanism proposed by Zitzewitz et al. (97), quantitative agreement can be achieved between the observed rate and monomer fractional helix content using the secondary structure prediction algorithm AGADIR (92).

One of the factors responsible for the lack of a systematic dependence of the folding rate constants on the thermodynamic driving force in the above systems is that different folding mechanisms may be operating. For example, the cold shock proteins studied by the Schmid group are β-sheet proteins in which the native-like transition state is arrived at by a more concerted, higher-order mechanism. The transition state would involve nonlocal interactions of the sort predicted by the nucleation-condensation model as opposed to a sequence of two-body collisions assumed for the diffusion-collision model used in the all-helical monomeric λ repressor. Thus, the bias toward stability of local structural units
is small in the former, owing to the unlikely stability of an isolated \( \beta \) strand or incomplete \( \beta \)-sheet, and considerably higher in the latter, helical protein. The bias toward stabilization of structural units by local versus nonlocal contacts has been addressed by Baker and coworkers (123) using a contact order parameter as discussed above. For the \( \beta \)-sheet proteins, the interplay between local and nonlocal interactions can also be rationalized using the diffusion-collision model if the isolated association-competent microdomains are relatively unstable (\( \beta \ll 1 \)) but achieve significant stability (\( \beta \sim 1 \)) on subsequent association [see Section VII,B and Eq. (3) for a definition and discussion of \( \beta \)].

B. Thermodynamic Parameters

1. Enthalpy, Entropy, and Heat Capacity

The relative contributions to the free energy barrier of the activation entropy, \( \Delta S^a \), activation heat capacity, \( \Delta C_p^a \), and activation enthalpy, \( \Delta H^a \), can be delineated by studying the folding and/or unfolding kinetics at various temperatures and denaturant concentrations. The thermodynamic contribution of the solvent, denaturants, and the polypeptide chain collapse to the free energy barrier can be assessed by these studies and complement equilibrium studies (150, 151) and kinetic work relying solely on denaturants (152). The analysis is carried out using the transition state formalism [Eq. (1)] and the temperature dependence of the Gibbs free energy:

\[
\Delta G^a (T) = \Delta H^a (T_o) - T \Delta S^a (T_o) + \Delta C_p^a \left[ (T - T_o) - T \ln \left( \frac{T}{T_o} \right) \right] (2)
\]

Additionally, a linear dependence of the thermodynamic variables on denaturant concentration and a temperature independent \( \Delta C_p^a \) are usually assumed (153–155). The values obtained for \( \Delta H^a \) and \( \Delta C_p^a \), unlike \( \Delta S^a \), are independent of the choice of preexponential factor in Eq. (1) if the temperature dependence of the prefactor is not significant (see below).

Schmid and coworkers have used this approach in their study of the kinetically two-state protein CspB from *Bacillus subtilis* (111). Consistent with their characterization based on denaturants and homologous proteins (110), the activated state is very native-like, exhibiting a heat capacity comparable (\( \sim 90\% \)) to the native state. This is easily discerned from the large curvature of the refolding Arrhenius plots and its nearly linear
dependence for unfolding. A particularly interesting finding from this study is the interplay between enthalpy and entropy in determining the barrier to folding (Figs. 4 and 5). At low temperatures the barrier is suggested to arise primarily from enthalpic contributions whereas at high temperatures the entropic term dominates (Fig. 5). The equilibrium thermodynamic properties are shown in Fig. 4 for reference. This phenomenon is attributed to the hydrophobic effect, wherein the decrease in entropy reflecting dehydration of hydrophobic residues compensates for the loss in chain entropy for folding at low temperatures but not at high temperatures.

The high degree of similarity between the native and transition state of CspB, based on the $\Delta C_p$ and $m$-value criterion, is relatively unique.
Fig. 5. The thermodynamic parameters of the transition state of CspB at various temperatures, illustrating the interplay between enthalpy and entropy: activation heat capacity (A), enthalpy (B), and entropy (C). Measurements were obtained at pH 7.0. [From Schindler and Schmid (111), with permission. Copyright 1996, Am. Chem. Soc.]
Other proteins in which similar evaluations have been carried out give values of $m^* / (m^* - m_c)$ and $\Delta C_p^\ddagger / \Delta C_p(eq)$ closer to $\sim 0.7$ (134, 152, 154, 156-158). Gloss and Matthews (158) have noted that estimates of the placement of the transition state obtained using $\Delta C_p^\ddagger / \Delta C_p(eq)$ are systematically lower than those obtained using the $m^*$-value criterion (111, 149, 156). The apparent inconsistency was suggested to arise from differences in the contribution of hydrophilic surface area burial to $\Delta C_p^\ddagger$ and $m^*$-value (158).

A common feature of the transition states of proteins studied using this methodology is a large activation enthalpy for unfolding. The values for CspB and $\alpha$TS are typical, in the range from $\sim 20$ to $25$ kcal·mol$^{-1}$ (111, 152). The disruption of hydrogen bonds has been proposed to give rise to this large value based on the argument that the enthalpy changes for transfer of nonpolar compounds to water is negative or only slightly positive at $25^\circ$C (152). Consistent with this reasoning is the known propensity of denaturants such as urea to bind to hydrogen bond donors and/or acceptors (e.g., the amide linkages in the backbone), which may become exposed in the transition state (152). Interestingly, the refolding activation enthalpy for a small $\beta$-hairpin model peptide in the absence of denaturant has been suggested to be negative (see Section VI.B).

The binding of denaturants is also reflected in the denaturant dependence of the entropic terms, $-T \Delta S^\ddagger$, which becomes increasingly positive at higher denaturant concentrations (Fig. 5) (111, 152, 158). In a study employing urea (152), this was attributed to the immobilization of urea when bound to the protein or possibly the reduction in the conformational degrees of freedom of the polypeptide induced by urea bound to two different sites.

In these studies, the temperature dependence of the preexponential factor [Eq. (1)] was assumed to be given by the Eyring equation. This formalism, however, does not take into account configurational diffusion across the folding barrier (159). Consideration of configurational diffusion has been proposed by Bryngelson and Wolynes to lead to a non-Arrhenius temperature dependence of the folding rate ($\ln(k) \sim 1/T^2$) that has its origin, not in the change in transition state heat capacity, $\Delta C_p$, but in the rate of escape from local minima on a rough energy landscape (159). In an effort to delineate the contributions of these two mechanisms to the non-Arrhenius behavior, Scalley and Baker analyzed the folding rate of protein L and CspB as a function of temperature at constant stability contours, $\Delta G/T$ (159). When the stability was taken into account by addition of denaturant, Arrhenius behavior was recovered (Fig. 6), including a similarity of activation energies. These results suggest that, at least in the range of temperatures studied (268 to
Fig. 6. Plots of the logarithm of refolding reaction rates of CspB versus \(1/T\) (A) and \(1/T^2\) (B) under constant stability conditions for CspB and protein L (159). Constant stability was maintained by addition of GdnHCL. The approximately linear dependence of the logarithm of the rate on \(1/T\) suggests that Arrhenius behavior is recovered after correcting for the stability of the native state. The linear relationship observed in both plots suggests that determining the contribution of configurational diffusion along a rough energy landscape may require a wider temperature range. [From Scalley and Baker (159), with permission. Copyright 1997, National Academy of Sciences, U.S.A.]
313 K), the temperature dependence of protein stability rather than configurational diffusion is the dominant factor. Arrhenius behavior under conditions of constant stability suggests that $\Delta C_p^0 / \Delta C_p = m^2 / m_{eq}$. One source for the slight deviations from linearity in constant stability Arrhenius plots (Fig. 6) may arise from differences between $\Delta C_p^0 / \Delta C_p$ and $m^2 / m_{eq}$, as has been suggested for several proteins (see above) (111, 149, 156, 158). An Arrhenius temperature dependence between 280 and 358 K was also observed for the folding kinetics of the N-terminal domain of ribosomal protein L9 when analyzed at constant $\Delta G / T$ contours (160). However, the slope of the stability corrected Arrhenius plot for this protein ($-8600$ K) differed from that of protein L and CspB ($-10,800$ and $11,000$ K, respectively). The physical interpretation for the differences in slopes awaits further study.

The temperature dependence of the viscosity of the solvent provides an additional, often unaccounted for, contribution to the temperature dependence of the refolding and unfolding rates. Several proteins for which a thermodynamic analysis has been carried out (e.g., $\alpha$TS and CspB) exhibit an inverse dependence of the folding or unfolding rate on the solvent viscosity. Because the viscosity of water varies in a nonlinear manner by approximately a factor of three over the temperature range of typical studies (e.g., $\eta_{273} = 1.787$ cp and $\eta_{313} = 0.6529$ cp), it would be useful to incorporate this factor in extracting thermodynamic parameters and in delineating non-Arrhenius contributions to the temperature dependence under constant stability conditions.

2. Pressure Studies

An increase in pressure is known to reversibly denature proteins, reflecting a reduced reaction volume of unfolded proteins (161–166). Recently, several groups have begun kinetic studies using pressure jumps to investigate the effect of pressure on refolding and unfolding rates (20, 165). Pressure studies have the advantages of fast triggering ($\sim 100$ $\mu$s), rapid equilibration, bidirectionality, high repetition rate, denaturant-free conditions, and the requirement of small sample volumes (20, 165). Additionally, pressure jumps ending in the equilibrium transition zone provide an opportunity to monitor both unfolding and refolding reactions. By relating the changes in the rates of folding and unfolding to activation volumes via transition state theory and using the thermodynamic relationship between free energy and pressure, $(\delta \Delta G^0 / \delta P)_T = \Delta V^0_T$, useful and unique information on the transition state can be obtained.

In their work on staphylococcal nuclease, Royer and coworkers found that increases in pressure (up to several kbars) result in decreased rates
(i.e., $\Delta V_T^c > 0$) of both folding and, to a lesser extent, unfolding (165). Similar observations were made on CspB by Schmid and coworkers in studies at pressures up to 160 bars (20). The decreased rate for refolding was suggested to reflect a significant role for dehydration of the protein in refolding (165). For unfolding, the activated state was likened to a swollen native-like protein with an increased internal void volume caused by disruption of tertiary contacts, referred to as a “dry molten globule” (19, 167-169).

In related studies of fluctuations in proteins at the single-residue level, Bryant and coworkers (170) investigated the hydrogen-deuterium exchange kinetics of individual amide sites in $^{15}$N-labeled T4 lysozyme at 22°C at pressures up to 200 MPa (~2 kbar). Their results suggest that there is no simple correlation between activation volumes and the secondary structural unit to which the residue belongs. Adjacent residues, for example, were found to exhibit different activation volumes. The generally small activation volumes were found not to be consistent with major unfolding events. These workers note the small energetic contributions, even at high pressures, made by the small activation volumes and emphasize caution in making mechanistic interpretations.

The relative contributions of internal and solvent friction, as might be reflected in the pressure dependence of the preexponential factor in Kramers’ theory, are not clear, and the possible shortcomings of the transition state formalism have been noted (61,170). In fact, the meaning of $\Delta V_T^c$ has been suggested to reflect not a direct physical volume change but a difference in the interaction of the reactants with the solvent environment (61). Simulations on ring flip reactions in BPTI suggested that pressure effects on the friction term in Kramers’ theory are not insignificant. This is consistent with the known pressure dependence of friction effects for simple condensed phase reactions, as illustrated in the isomerization kinetics of cyclohexane (63). The quantitative interpretation of the volume changes in the transition state is also likely to be influenced by consideration of compressibility effects on the protein itself (166). Despite these complications, the emerging area of pressure studies on folding kinetics offers significant opportunity for insights into the nature of barrier dynamics in folding reactions.

C. Kinetic Isotope Effects

Studies of kinetic isotope effects induced by substitution of protium for deuterium enable a test of the role of the strength of hydrogen bonding in protein folding transition states and barrier dynamics. As has been pointed out in several recent studies, delineation of the precise
contributions to these phenomena in protein folding requires consider-

ation of various factors related to the concomitant changes introduced
in protein-protein, protein-solvent, and solvent-solvent interactions when
\( \text{H}_2\text{O} \) is replaced by \( \text{D}_2\text{O} \) as the solvent (171-173).

In a study on hen egg-white lysozyme, Itzhaki and Evans examined a
number of these contributions to the three-phase refolding kinetics
(173). The slower observable phase had an approximately 50% faster
rate in \( \text{H}_2\text{O} \) than \( \text{D}_2\text{O} \) although similar rates were found for the faster
observable phase. These results were not due to the isotopic nature of
the backbone amide hydrogens, differences in viscosity between \( \text{H}_2\text{O} \)
and \( \text{D}_2\text{O} \), or uncertainties in the \( \text{pH} \) measurements. At low \( \text{pH} \), a faster
rate in \( \text{D}_2\text{O} \) was observed, indicating that the ionization of titratable
groups is an additional factor to be considered in the analysis of the
kinetic isotope effect. The opposing effect at high \( \text{pH} \) was attributed to
an isotope dependence of the hydrophobic effect.

Consistent with these findings, the folding kinetics of the SH3 domain
of PI3 kinase at \( \text{pH} 7.2 \) exhibit a rate enhancement of 1.6 in \( \text{D}_2\text{O} \) relative
to that in \( \text{H}_2\text{O} \) for the fast folding kinetic phase (174). However, the
slow kinetic phase, corresponding to cis/trans proline isomerization
between unfolded states, did not show an isotope effect.

Augmented solvent-solvent interactions and a greater hydrophobic
effect in \( \text{D}_2\text{O} \) was inferred from a stabilization of kinetic intermediates
in \( \text{D}_2\text{O} \) relative to \( \text{H}_2\text{O} \). The effect was proportional to the \( \text{m} \)-values of
the intermediates, and hence to the exposure of nonpolar surface area
(171). Delineation of the enthalpic and entropic contributions to the
rate enhancement in \( \text{D}_2\text{O} \) has not been addressed in kinetic studies in
a manner analogous to equilibrium studies (172).

D. Viscosity Dependence

A common feature of various models of protein folding kinetics in-
volves diffusional motion of the unfolded polypeptide chain as the reac-
tion proceeds toward the native state. This may occur, for example, in
the form of a collapse process (38, 175) or diffusion of preformed
structural units (74-76, 176). Viscogens have been employed by several
workers in an effort to determine the role of diffusion in the barriers
to folding and address the role of the solvent. Although early studies in
this area were characterized by conflicting reports on the effect of visco-
gens (177, 178), these studies did not include the effect of viscogens on
protein stability in their analysis (179).

Chrunyk and Matthews studied the single-exponential unfolding reac-
tion of the \( \alpha \)-subunit of tryptophan synthase as a function of glycerol,
glucose, and ethylene glycol and found a direct linear relationship, with a slope of unity, between the fractional change in unfolding time constant, $\tau/\tau_0$, and the fractional change in solvent viscosity, $\eta/\eta_0$ (179). The stabilizing effect of the viscogen was accounted for by comparing data at constant stability between folded and unfolded states. A viscosity dependence of the rate was not observed for the polymer polyethylene glycol at comparable viscosities, illustrating differences in microviscosity and macroviscosity. The steric exclusion of the MW = 8000 polyethylene glycol from the vicinity of the protein was suggested to not affect the hydration of the protein surface and, therefore, the rate-limiting opening of this $(\beta\alpha)_2$-barrel protein. A molecular size dependence of the effect of viscosity has been corroborated in other studies (180). The complex four-channel folding mechanism postulated for the $\alpha$-subunit of tryptophan synthase (31, 181) precluded a similar study of the refolding reaction.

Schmid and coworkers considered the effect of surface tension, dielectric constant, and viscosity on the refolding and unfolding kinetics of the kinetically two-state protein CspB (182). Only viscosity, which showed a unitary linear relationship between $\tau/\tau_0$ and $\eta/\eta_0$, exhibited a correlation with the folding rate at constant stability (Fig. 7). The transition state of this protein is very native-like (20, 110, 111) and the viscosity

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Fig. 7. Viscosity dependence of the refolding (left panel) and unfolding (right panel) reaction of CspB. The viscosity was varied by addition of sucrose at 25°C. The rates, normalized to the rates in the absence of viscogen, were compared under conditions of constant stability of the native state, achieved by addition of GdnHCl. [From Jacob et al. (182), with permission. Copyright 1997, Am. Chem. Soc.]
dependence was attributed to the influence of solvent friction on the chain dynamics in the activated state.

The viscosity dependence of the second order refolding reactions of two dimeric proteins, Arc repressor (114) and GCN4-p1 (183), has also been studied. Both of these proteins and their mutants exhibit refolding rates less than that expected for a diffusion-limited reaction. Nonetheless, for GCN4-p1 and wild-type Arc repressor a $1/\eta$ viscosity dependence of the rate is observed. However, the MYL mutant of Arc repressor, in which a buried salt-bridge is replaced with a cluster stabilized by the hydrophobic effect, exhibits no viscosity dependence of the kinetics; the folding rate of the MYL mutant may even accelerate at higher viscosity. This behavior was attributed to the presence of a high-energy (i.e., not well-populated) dimeric intermediate for both and a change in the rate-limiting step for the more stable MYL mutant. In the wild-type Arc repressor, the rate-limiting step is the association of monomers to form the high-energy dimeric intermediate. In the MYL mutant, it is the interconversion of the dimeric MYL intermediate to the dimeric native form. The latter step is apparently independent of viscosity, although corrections for the stabilizing effect of the viscogen sucrose were not considered.

In analyzing the effect of viscosity on the folding kinetics of aTS and CspB, a constant free energy difference between reactant and product states was achieved by comparing the rates in the presence of appropriate amounts of denaturant to counteract the stabilizing effect of the viscogen (179). This approach, also utilized in the analysis of the temperature dependence of folding kinetics (159), has recently been criticized by Ladurner and Fersht (184), who suggested that viscogens and denaturants affect stability by different mechanisms. It was postulated that the compensation of the native state stability in this manner may unpredictably affect the transition state energy. Further, the observation of a viscosity effect for the relatively native-like, compact transition states in aTS and CspB was suggested to lead to a paradox: If the viscosity dependence reflects chain diffusion, how could a viscosity dependence exist for transitions between states with nearly native compactness that have already excluded solvent?

A number of questions concerning the influence of viscosity on folding would benefit from further study. The magnitude of the differential stabilization of the transition state relative to the native state is not known in the studies on aTS and CspB, and this may be a source for the nonlinearity observed at high concentration of viscogen. However, deviations from Kramers' behavior in the high friction regime have also been observed in small molecule systems (37, 55). A systematic study of the
stabilizing effect of osmolytes suggests that the opposing effect on protein stability of osmolytes and urea is largely additive (185). Additionally, the validity of the premise that the viscosity should have minimal effect on transitions between compact states (173, 183, 184) is not established. For αTS, a non-negligible amount of hydrophobic surface area [corresponding to exposure of ~50 residues based on m^2-values (31)] is exposed to solvent in the transition state. Similarly, studies on different strains of CspB have shown that surface residues do not have native-like interactions in the transition state. Although a transition state may be very native-like, based on ΔC° and m^2-values, the significant and nonzero values of these parameters suggest that solvation of at least parts of the protein must occur in the transition state.

The treatment of the solvent as uncoupled from these barrier dynamics is open to skepticism, especially considering the established role of viscosity not only in protein dynamics but also in condensed phase reactions such as isomerization reactions in aprotic solvents (63), solvation dynamics, proton transfer, electron transfer, and enzyme catalysis reactions (37). The frictional effect of viscosity, as given in Kramers' treatment, can have various contributing factors and is not necessarily simply related to diffusion associated with bimolecular reactions (44, 61). A significant effect of solvent viscosity on even internal protein dynamics was demonstrated in a combined temperature and viscogen study on CO dynamics in hemoglobin (59). Further, consistent with the m^2 and ΔC° values of many folding reactions, molecular dynamics simulations (40, 169, 186) and unfolding temperature-jump studies on the picosecond/nanosecond time-scale (15) implicate a role for water penetration into the protein. Considering that increasing viscosity leads to damping of displacement motions and side chain motions (61), it is not unreasonable to expect a viscosity dependence for even native-like transition states in unfolding. The basis for the effect on displacement motions was suggested to be due to the comparable time-scale of these motions (~150 ps) to those of water (~40 ps). However, protein dynamics may be coupled to the solvent dynamics in a more complicated fashion, as in the hierarchically constrained dynamics model applied to myoglobin (187). Thus, a wide range of studies suggest that a paradox need not be invoked to account for the viscosity dependence of transitions between collapsed states.

It is noteworthy that the viscosity dependence of the rate in the studies on αTS and CspB follows a 1/η dependence, where η ~ 1. In the study on CO-hemoglobin, a value of η < 1 was found for CO dynamics. The subunitary value was attributed to attenuation and transformation of the effect of the solvent on internal protein dynamics.
Similar observations have also been reported for other systems (188). A distinction between internal friction and solvent friction was used in the interpretation of the conformational relaxation of myoglobin (62). The refolding kinetics displayed a Kramers-like viscosity dependence only at high viscosities (~20 cp). The analysis employed partitioned the friction term into an internal and solvent friction component via the relation $1/\eta' = 1/(\eta + \sigma)$, where $\sigma$ is the contribution from internal friction. In contrast, early refolding dynamics of apomyoglobin display a $1/\eta$ viscosity dependence in the nanosecond/microsecond time-scale, consistent with a solvent exposed collapse process (189). Lattice model simulations indicate that internal friction is not significant in cases where the native structure is directly accessible from relatively open structures (71, 190). Internal friction was significant only in transitions from compact misfolded states to the native state. A value of $\kappa \sim 1$ for protein folding and unfolding reactions might suggest that, at least in these systems, the role of solvent and the effect of viscosity is more direct, possibly via damping of fluctuations of solvent accessible residues. To what extent these fluctuations and solvent dynamics are coupled to folding and unfolding reactions will hopefully be further addressed with the recent focus on the effect of viscogens.

A significant effect of viscosity is also observed in the folding kinetics for several model systems. The folding kinetics of the 16-residue $\beta$-hairpin (41) (see Section VI,B) displayed a $1/\eta$ dependence whereas the kinetics of $\alpha$-helix formation in a 17-residue peptide (17) showed a $1/\eta^\kappa$ dependence with $\kappa \sim 0.6$ (191). A plateau at low viscosities, as might be expected for an internal friction component, was not observed in the latter study, presumably because of the availability of a limited range of viscosities that did not significantly affect the stability of the helical peptide. The folding kinetics of a simple dimeric coiled-coil $\alpha$-helical protein GCN4-p1 (see Section V,A), however, show a $1/\eta$ viscosity dependence independent of whether the subunits are tethered (unimolecular reaction) or untethered (bimolecular reaction) (183). These findings, $\kappa = 1$ for GCN4-p1 and $\kappa \sim 0.6$ for $\alpha$-helix formation, may be viewed as consistent with the presence of helical structure in the transition state of GCN4-p1, indicating that $\alpha$-helix formation is significantly faster than the rate of collisions between association-competent monomeric conformers. A disparity in time-scales between collision frequency and $\alpha$-helix formation in both the tethered and untethered GCN4-p1 species may possibly mask the fractional viscosity dependence of $\alpha$-helix formation.
E. Placement of the Transition State

With the assumption that a reaction coordinate along a particular order parameter is appropriate for the visualization of the folding reaction, several studies have aimed toward obtaining a description of the shape of the reaction coordinate and location of the transition state. As discussed above, the location of the transition state along the reaction coordinate is most often expressed in terms of the refolding and unfolding kinetic $m$-values, $m_f$ and $m_u$, respectively, relative to the equilibrium $m$-values (192, 193). Although point mutations and denaturant typically do not significantly affect the value of $\alpha = m_f / (m_f - m_u)$, several proteins have shown a significant dependence of $\alpha$ on point mutations. For example, the change in the kinetic $m$-values for point mutations in Arc repressor was interpreted to suggest that the folding barrier was not smooth but broad with local, high-energy minima. The drastic decrease in $\alpha$ thus reflects a switch in the peak barrier height from a late transition state to an early one (114). Although a similar argument may be applied to the effect of two Ala/Gly mutations in monomeric $\lambda$ repressor (see Section VII,B), the changes in this case were suggested to arise from variation in the number and nature of folding pathways (4, 5).

Broad folding free energy barriers have also been invoked by Oliveberg and coworkers to rationalize curvature in refolding and unfolding sides of chevron plots. The barrier is viewed as relatively flat, with a peak position along the reaction coordinate that is dependent on the solvent conditions (194–196). Thus, the position of the transition state along the reaction coordinate is different in refolding and unfolding reactions. To what extent this is a general phenomenon is an unresolved question, and alternative explanations, such as the presence of intermediates, are difficult to rule out. A global analysis of the raw kinetic data from which chevron plots are obtained (31, 197), particularly with multiple probes, may be useful in this regard. Also, the extent to which local minima along a broad barrier affect the preexponential term of the rate expression [Eq. (1)] is not clear. Dissecting this contribution from a gradual movement of the transition state along the reaction coordinate may not be straightforward in that it may respond similarly to point mutations. Presumably, other possible sources of nonlinearities that may affect the symmetry of chevron plots, such as the nonlinear dependence of activity (26) and viscosity (see Section V,D) on denaturant concentration, are not significant.

F. The Role of Folding Intermediates

The increasing number of examples of proteins that are capable of folding without obligatory intermediates (3) has raised questions regard-
ing the role of populated intermediates in folding (6–8, 198). Two-state folding proteins usually contain fewer than 100 amino acids and exhibit folding rates that vary by several orders of magnitude (3). In contrast, many larger proteins fold by a multistate mechanism, typically populating the native state on a longer time-scale. Two opposing views of the kinetic implications of populated folding intermediates have evolved. One view postulates that, within a classical kinetic scheme, intermediates slow down the overall folding rate even though the height of the free energy barrier relative to the unfolded state has not changed (198). The alternative view holds that this is physically unrealistic if folding intermediates serve to direct the folding reaction pathway (6). The narrowing of the conformational search space by the populated intermediates is interpreted as enhancing the formation of the native state.

The kinetic argument for the slowing down of a folding reaction by intermediates is illustrated in Fig. 8A. For a three-state reversible reaction in which there is a rapid prior equilibrium between U and I, the folding rate is dependent on the relative stability of I. For example, $\Delta G_{UI} = 0$ results in a slowing down of the folding rate by a factor of two relative to a two-state reaction with the same transition state free energy barrier height, $\Delta G_{UN}^\dagger$. The effect of the intermediate is manifested in the prefactor in Eq. (1). One may equivalently visualize this as an entropic stabilization of the unfolded state. With regard to mutational analysis, in the limit that the stabilization or destabilization of the intermediate can be regarded as a small perturbation of the reaction coordinate profile, the overall folding rate (rate of formation of the native state) is not affected. This occurs because $\Delta G_{IN}^\dagger$ is compensated by the increased population of I, via $\Delta G_{UI}$. This argument assumes, however, that destabilization or stabilization of I, $\Delta G_{UI} = -\Delta G_{IN}$, is independent of $\Delta G_{UN}^\dagger$ and $\Delta G_{UN}^\dagger$. In effect, stabilization of I is derived from non-native interactions. This behavior has been suggested as unlikely for an obligatory intermediate (6) and would more logically reflect the presence of an off-pathway intermediate.

The interdependence of $\Delta G_{UI}$, $-\Delta G_{IN}$, and $\Delta G_{UN}^\dagger$ is illustrated by the folding kinetics of the relatively simple dimeric helical coiled-coil peptide, GCN4-p1. This peptide folds by an apparent two-state mechanism, $2U \rightleftharpoons N$, with a bimolecular rate constant at least three orders of magnitude less than that of a diffusion controlled reaction (97, 98). As described above, the folding transition state is dimeric and the folding rate correlates with the fractional helix content of the monomers as discerned from mutational analysis (J. Zitzewitz, personal communication). Thus, an increase in the stability of an intermediate, partially helical monomer, gives rise to an increase in the overall folding rate.
Fig. 8. Schematic protein folding reaction profiles in the presence of an intermediate state (I) of varying stability. The relative stability of the native state $\Delta G_{\text{NN}}$ is assumed to be constant. The folding rate (rate of formation of native state, N) is obtained by solution of the eigenvalue equation corresponding to a reversible three-state kinetic model, $U \rightleftharpoons I \rightleftharpoons N$. In panel (A), the stabilization of the intermediate does not affect $\Delta G_{\text{UTS}}$. In this scenario, the folding rate decreases with increasing stabilization ($\Delta G_{\text{II}} < 0$) of the intermediate relative to the unfolded state, U. The stability of the intermediate increases progressively from the dashed to the solid line. In an alternative view shown in the lower panel (B), stabilization of the intermediate also affects the transition state free energy, $\Delta G_{\text{UTS}}$, and, therefore, the folding rate is enhanced with increasing stabilization of the intermediate. The figure shows the case in which stabilization of the transition state, $\Delta A G_{\text{UTS}}$, is equal to stabilization of the intermediate, $\Delta A G_{\text{II}}$. In most cases, a preferential stabilization of the transition state ($-\Delta A G_{\text{UTS}} > \Delta A G_{\text{II}}$) (i.e., an even greater enhancement in rate) would be expected. The latter case more closely describes results obtained for GCN4-p1, where U represents the unfolded monomers, I corresponds to the partially helical monomers, and N represents the dimeric coiled-coil. The transition state (TS) is dimeric with some helical structure (97, 99).
This is illustrated schematically in Fig. 8B. In contrast with the above assumption and consistent with previous suggestions (6), stabilization of the intermediate, $\Delta \Delta G_{\text{UI}} < 0$, also results in stabilization of the transition state, $\Delta \Delta G_{\text{UN}} < 0$. This behavior is consistent with the proposal that partially helical monomers are obligatory intermediates that contain native-like interactions (97, 99).

In a recent review of folding intermediates (6), it was noted that the only instances in which destabilization of an intermediate increases the folding rate is when the intermediate is a kinetically trapped species with non-native interactions (6) [e.g., non-native proline isomers (29) or non-native disulfide bonds (30)]. This observation and the present discussion support the view of folding intermediates as being analogous to microdomains in the diffusion-collision model: structures in which some fraction of nativelike interactions have been formed that serve to limit the conformational search space and thus enhance the folding rate.

Even for systems with two-state folding kinetics, high-energy intermediates ($\Delta G > 0$) may affect the magnitude of the apparent free energy barrier. Thus, deciphering specific contributions to the folding rate and apparent transition state free energy may require consideration of the profile, or shape, of the reaction coordinate. The role of high-energy intermediates along the reaction coordinate, as might be inferred from mutational studies (4, 114), may be significant in these kinetically simple systems. Measurement of the absolute free energy of the transition state is hindered by uncertainties in the preexponential factor of the rate equation [Eq. (1)].

VI. ELEMENTARY EVENTS AND MODEL SYSTEMS

A. Helix/Coil Transition

Laser-induced temperature-jump techniques, combined with spectroscopic methods capable of monitoring structural changes on the nanosecond time-scale, and the discovery of considerable helical content in small peptides have led to recent measurements of helix-coil transition rates in small peptides (16, 17, 199). These studies are an important advance over most earlier studies on homopolymers because the helix-coil interconversion can be directly monitored and also because the 21-residue peptide used (200, 201) is considered to be a more appropriate model for $\alpha$-helices in protein folding reactions than the homopolymers in nonaqueous solvents (202).
Williams et al. (16) probed the helix-coil kinetics using the infrared amide I absorption band of the polypeptide backbone at 28°C following temperature jumps of ~18°C. The measured unfolding relaxation time constant \( \frac{1}{k_{\text{obs}}} = \frac{1}{(k_f + k_u)} \) of 160 ns and the estimated equilibrium constant, \( K = \frac{k_f}{k_u} \), were used to calculate a folding rate constant, \( k_f \), of approximately \( 6.3 \times 10^7 \text{s}^{-1} \). An ultraviolet resonance Raman investigation of this peptide (199) yielded a comparable relaxation time of 180 ns in temperature jumps from 4 to 37°C. However, different refolding (\( k_r = 9.1 \times 10^5 \text{s}^{-1} \)) and unfolding (\( k_u = 5 \times 10^6 \text{s}^{-1} \)) rates were calculated, presumably reflecting different estimates of the equilibrium constant for the helix-coil transition. The temperature dependence of the refolding rate, \( k_r \), was examined by Lednev et al. (199) and found to exhibit a negative activation energy. A negative activation energy for β-hairpin formation has been interpreted as indicative of an entropic barrier (203) (see below).

A complementary study using an N-terminal fluorescent probe suggested a considerably faster helix-coil transition rate (17). The nearly eight-fold faster rate in the fluorescent study was explained in a kinetic helix-coil zipper model as arising from differences in the probes. The infrared absorption monitors the average helical content whereas the fluorescent label reports on the N-terminal dynamics. The single-sequence homopolymer kinetic zipper model suggested that the fast phase observed by fluorescence monitors redistribution of helical lengths whereas the amide I absorption kinetics reflect traversal of the dominant barrier in the helix-coil transition. Follow-up studies, in which the N-terminal probe was replaced with a tryptophan and the \( i + 4 \) position with a histidine, led to diminished fraying at the N-terminus and kinetics similar to the infrared probe (204). A kinetic zipper model extended to heteropolymers was also able to explain these experimental observations, although a modest discrepancy in the relative amplitudes of the fluorescence signal was noted.

The temperature dependence of the helix-coil transition was also studied and modeled using the homopolymer kinetic zipper model (17). A minimum in the logarithm of the rate as a function of temperature near the melting temperature was observed for both the average helical content and the N-terminal dynamics. This nonmonotonic dependence was also evident in the zipper model and attributed to the temperature dependence of the rate of equilibration between helix-containing species.

The observed helix propagation rate deduced from the analysis, \( \sim 1 \times 10^8 \text{s}^{-1} \), is nearly two orders of magnitude slower than that observed for many homopolymers in nonaqueous solvents (202). The slower rate
in water has been suggested to reflect the necessity of breaking hydrogen bonds with the solvent although molecular dynamics simulations suggest that this process can proceed without a barrier in water (205).

B. β-Turn Formation

An elegant illustration of the nature of barriers in an elementary folding reaction was given by the analysis of the folding of a simple β-hairpin peptide (41, 204). The folding kinetics of the 16-residue peptide, monitored by tryptophan fluorescence and excitation energy transfer between a tryptophan and a dansyl label, exhibits many features analogous to the folding of proteins, such as rapid (~1 μs) two-state kinetics, an entropic barrier, and a funnel-like rough free energy surface. The entropic contribution to the free energy of the barrier was delineated by the negative activation energy, −1 kcal mol⁻¹. In the statistical mechanical model developed by the same group (203), stabilization of the native structure is a result of the “hydrophobic interactions” in a cluster of four side chains. The transition state structure is attributed to the entropically unfavorable formation of native dihedral angles (and appropriate hydrogen bonding interactions) for the seven central peptide bonds. Crossing of the free energy barrier is accomplished by the stabilizing effect of side chain interactions in the hydrophobic cluster. Unlike helix propagation, extension of the β-turn by an additional residue in the absence of side chain interactions is energetically unfavorable in the model. This prediction was confirmed by the observation of a faster folding rate in a peptide in which the hydrophobic cluster was moved closer to the β-turn. The recovered parameters for the model were also used to suggest that the rate of peptide bond formation in both α-helices and β-turns is ~1 ns⁻¹. The slower rate of folding of β-turns (~1 μs versus ~100 ns for an α-helix) was suggested to reflect the greater number of nucleation sites in an α-helix. β-turns in this peptide model can only form at positions that permit the concerted formation of a minimum number of interstrand hydrogen bonds and the fluorescence-detected hydrophobic cluster in a productive folding nucleus. Direct detection of the kinetics of hydrogen-bond formation in this peptide has not been reported.

C. Loop Formation

In many of the phenomenological folding models, the coalescence of either key residues or microdomains plays a critical role. The length of the flexible intervening loop regions have been shown in some studies (see Section IV, D) to correlate with the height of the free energy barrier.
Rates of loop formation in a random polypeptide chain have therefore been proposed as an indicator of an upper limit of how fast a protein can fold (54, 64).

This hypothesis was investigated (54, 64) by studying the rate of intramolecular ligand binding between methionines and the heme in reduced cytochrome c under denaturing conditions. By establishing the diffusion-limited binding of extrinsic methionine under similar conditions to a model heme containing peptide, and with a knowledge of the sequence length of the loop, calculations using polymer theory were used to extrapolate the maximum folding rate of a typical loop in a protein (~10–12 residues). The 40-μs time constant for formation of the 50 residue heme-methionine loop resulted in an extrapolated time constant of ~1 μs for a loop of approximately 10 residues. Although independent tests of this value have not been carried out, similarity between this maximal rate and kinetics of β-hairpin formation has been noted (204). Consistent with the proposal, the fastest measured folding rate for a globular protein at present is the M37 variant of the 80 residue monomeric λ repressor, which folds in ~10 μs (5). Additionally, MD simulations in which a nativelike partially folded structure is attained within ~1 μs (124) suggest that once a stable contact (loop-closure) is accomplished, folding, or condensation, is rapid, more directed, and possibly cooperative.

Results from mutational analysis of the loop regions of several proteins are qualitatively consistent with the above findings. Insertion of variable linker regions gives rise to a decrease in the folding rate (26, 206) and disruption of β-hairpins (105) also results in a pronounced decrease of the refolding rate (see Section IV,D).

D. Chain Collapse

When a folding reaction is initiated by a sudden change in the solvent, the earliest events are thought to reflect the adjustment of the polypeptide chain to the new conditions. This adjustment may reflect, for example, a rapid dilution of denaturant, a temperature jump, or a pressure jump. The redistribution of denatured conformations in the new environment is expected to be rapid and unresolved in many studies. Stopped-flow refolding studies often display a “burst-phase” species that forms in the sub-millisecond regime. Recent experiments on faster time-scales have addressed the physical origins of this behavior, although a consensus has not been reached.

In rapid-mixing denaturant dilution refolding experiments with an ~100-μs dead time, the extent of intrinsic tryptophan fluorescence
quenching by the heme in reduced cytochrome c was taken as a gauge of compactness (64). At the earliest observation time, a significant quenching was observed whose amplitude exhibited a nonlinear denaturant dependence. This response was interpreted as reflecting the adjustment of the unfolded polypeptide toward more compact conformations under non-denaturing conditions. Sosnick et al. (50) have interpreted burst-phase stopped-flow CD signals in several proteins along similar lines. In continuous-flow microsecond mixing studies on cytochrome c (49), however, an exponential response of the tryptophan quenching with no burst-phase signal was observed. This result suggests the presence of a distinct barrier and a time-scale for collapse commensurate with folding. Whether these phenomena are simply an effect of the solvent and proceed without a barrier or represent a specific folding event with a barrier is not clear at present. The observation of an exponential response in a direct measurement of the dynamics, however, is preferable to making inferences from the denaturant dependence of the burst-phase amplitude in resolving this issue. Delineating the dominant contributions to the barrier for chain collapse (i.e., enthalpic versus entropic) may yield additional insights. Measurements of compactness on the μs time-scale in the refolding from denaturant-induced unfolded states of other proteins have not been carried out.

The early events in the folding of apomyoglobin from the cold denatured state have been monitored by intrinsic tryptophan fluorescence following laser-induced temperature jumps (~0 to 22°C and -7 to 10°C) on the nanosecond time-scale (40, 189). The collapse of the largely unfolded coil state to a complex with nativelike contacts for the AGH helices occurred in a biphasic manner. A fast phase (~250 ns) was attributed to a local change in the environment of the tryptophans, and the slower phase (~5–10 μs) (18, 189) was associated with global collapse. A viscosity dependence consistent with that expected for a collapse was observed.

A complementary laser-induced temperature-jump study by Gilman-shin et al. on apomyoglobin (47) monitored the amide I infrared absorbance band of the backbone on the 10-ns to 100-μs time-scale. This absorption probes the C=O stretching vibrations of the backbone carbonyls and permits distinction between a random coil, solvated α-helix and an α-helix with nativelike tertiary contacts (47). In jumps of ~+15°C to a final temperature of 60°C, where both unfolding and refolding take place, formation of secondary structure (α-helices) occurred nearly three orders of magnitude faster than tertiary contacts (48-200 ns versus ~280 μs). These results suggest that collapse may be mediated by even minute populations of helical structure. Winding of the bipolar patterning of residues into a helix would lead to the development of
hydrophobic surfaces that might be large enough to stabilize a productive complex.

VII. COMPARISON OF COMPUTATIONAL AND EXPERIMENTAL RESULTS

A. Molecular Dynamics Simulations

Comparisons of molecular dynamics calculations with experimental folding studies have been limited in two significant respects. First, the durations of the simulations were significantly shorter than the timescales of the experimental methods. Second, the statistics of the folding or unfolding reaction were difficult to attain because a significant computational effort was required for a single trajectory. Within the past year, however, reports of studies overcoming these limitations have started to appear. The results have been instrumental for elucidating the molecular details of folding transition states for real proteins, for validating the mutational analysis approach, and for demonstrating the presence of "pathways" in folding reactions.

An investigation of the structural characteristics of a transition state was carried out on Cl2 by Li and Daggett (169, 207) using unfolding molecular dynamics simulations (208). The unfolding transition state was identified by analysis of the conformational changes between the structures in the unfolding trajectory. Free energy minima tend to appear as clusters in this analysis and the region between the first two clusters, 220–225 ps, was taken as the transition state ensemble (Fig. 9, top panel). A prominent feature of the average structure representing this time-window is an expanded hydrophobic core (Fig. 9, lower panel). Water, which was included explicitly in the simulation, does not occupy the additional volume. Similar structural features (i.e., where the tertiary contacts are disrupted and side chains are dynamic but the void space is not occupied by water) are reminiscent of the transition state structure inferred by Royer and coworkers, referred to as a "dry molten globule," from unfolding/refolding measurements of staphylococcal nuclease at elevated pressures (19). Close agreement is found between the average transition state structure of Cl2 in the simulation with previously proposed structural features based on mutational analysis. Protein engineering studies of this protein (72, 209) had previously identified the transition state as having a nearly fully formed α-helix and a partially formed β-sheet, composed of strands 2 and 3. The φ-values obtained from the MD simulation, assuming that the change in free energy of the transition state is proportional to the number of contacts for the mutated residue,
agreed closely with experimental values, including the anomalous $\phi > 1$ result for V38A. Thus, in addition to the structural and dynamic insights, these results also reaffirm the mutational analysis approach (210, 211).

The statistics of the unfolding of CI2 were addressed by Lazaridis and Karplus in a study employing implicit water and multiple unfolding
trajectories (212). By conducting 24 unfolding simulations, these workers demonstrated that multiple unfolding trajectories exist, as might be expected from the so-called new view of protein folding. However, the simulations also revealed a preference for certain "pathways," suggesting that a specific order of events exists for the efficient folding of a protein. The simulations are also broadly consistent with a nucleation-collapse mechanism in which the rate-limiting step is the burial of a hydrophobic core via contacts made by the helix, strand 1 and other residues distant in sequence, possibly including the β3-β4 sheet (72).

Although the utility of unfolding simulations under conditions favoring the unfolded state as a probe of protein folding has been questioned (186, 213), the emergence of certain pathways in the above simulations places a range of experimental results on firmer ground. For example, mutational analysis and hydrogen-exchange pulse-labeling NMR of folding intermediates had previously suggested a preference for and persistence of certain structural features in the refolding of various proteins. The molecular dynamics simulations establish the correlated nature of these structural features, a point insufficiently demonstrated experimentally and previously argued as necessary for establishing the existence of pathways (33). Although the close correspondence between experimental ϕ-values found in the molecular dynamics simulations has been impressive, Baker and coworkers have suggested that comparisons against proteins with structurally polarized transition states, such as SH3 domains, may serve as a more stringent test (105).

Several recent molecular dynamics simulations of the refolding of proteins have revealed many features consistent with experimental findings (40, 124, 186, 214, 215). Although the time-scale of refolding simulations is currently short of that necessary for reaching a fully formed native state, characteristics of the transient conformations observed include a burst-phase hydrophobic-driven collapse, significant secondary structure formation, and nativelike topology with non-native side chain orientations. These studies have also indicated a bias toward certain preferred routes toward the native structure, consistent with findings of the unfolding simulations (124, 215). In a simulation extending out to 1 μs on the villin headpiece subdomain, HP-36 (124), for example, multiple pathways characterized by shallow barriers were found in the early stages of folding but only two pathways were dominant in leading to the metastable intermediate state. Cluster analysis also identified the formation of helical structure in two of the native helices as the initiation site for the folding of this protein. The refolding studies thus far show promise in bridging computational and experimental results based on their ability to reproduce many central features of folding reactions.
B. Diffusion-Collision Calculations

An extensive overview of comparisons between experimental data and calculations based on diffusion-collision theory has been given by Karplus and Weaver (75). Since this work, however, the access to very short timescales for monitoring early folding events has contributed valuable data on folding rates. Thus, a brief summary of several aspects of the diffusion-collision model and a discussion of recent studies in which calculations based on this model have been tested against direct measurements of sub-millisecond two-state folding rates seems justified.

The coalescence of two microdomains is estimated in the diffusion-collision model by the relation

\[ \tau \equiv \frac{1}{\beta} \frac{\Delta V}{DA} \]  

(3)

where \( l \) is the average of the inner and outer shell radii, \( \Delta V \) is the diffusion space defined by the region between the inner and outer shell radii, \( D \) is the diffusion coefficient, and \( A \) is the surface area of the inner shell (74, 75). The term \( \beta \) in Eq. (3) represents the probability that the two coalescing microdomains are in the correct conformation for successful coalescence. The isolated structures are taken to be static and the dynamics of the encounter complex are not considered. The back reaction is taken into account by consideration of the accessible surface area buried by the coalescence of two microdomains and the change in free energy per unit area (216, 217). The solution to the set of differential equations yields the time dependence of the population of all microdomains and the final structure (216).

One of the sources of uncertainty in these calculations is the estimation of \( \beta \). Most studies have assumed equal probabilities for correct elementary microdomain populations and for microdomains at later stages of folding (216, 217). Although this assumption may be appropriate in certain designed systems, its application to naturally occurring proteins is doubtful. Although Karplus and Weaver suggested the possibility of determining \( \beta \) experimentally by studies of peptides and fragments (75), its realization has not been straightforward. Recent progress in refinements (89–91) and parameterizations of helix-coil theory (92–94) have led to steps in this direction by permitting more reliable estimates of this parameter.

A central aspect of the study on monomeric \( \lambda \) repressor by Burton et al. (5) was the use of the secondary structure prediction algorithm AGADIR (92) in combination with mutational analysis and direct mea-
measurements of the refolding rate by NMR linewidth measurements (218). Their calculated rates using the diffusion-collision model for a series of Gly → Ala surface mutations give remarkable consistency with experimental measurements.

The results of the calculations also illustrate the manner in which mutations may change the folding mechanism or pathway. Their observations for the wild-type protein reveal that relatively few pathways are significant. This is in contrast to calculations on a designed four-helix bundle with identical helices in which "jigsaw"-like interconnections between various microdomains was observed (217). The differences presumably arise from the greater variations in stability of the elementary microdomains in the wild-type protein, and possibly other naturally occurring proteins, relative to designed sequences. Similar to the designed four-helix bundle, double Gly → Ala mutants of monomeric λ repressor have been proposed to yield no well-defined pathways to the native state.

The absence of a structurally specific rate-limiting event has been suggested to illustrate the shortcomings of visualizing the barrier via a reaction coordinate diagram picture. Additionally, if, as the simulations suggest, a number of free energy barriers of slightly different height are present, it is not clear why simple two-state behavior and single-exponential kinetics are observed. Using a reaction coordinate diagram, the change in the dominant pathway(s) upon mutation may be visualized as a change in the transition state along one or more order parameters. This hypothesis is depicted in Fig. 3. A similar mechanism has been used to explain the changes in denaturant dependence of the folding rate in Arc repressor (106).

VIII. Future Directions and Trends

A. Back to the Future

One of the striking observations in early investigations of protein folding reactions was the cooperative nature of the folding transition (193). Further studies, however, revealed that folding often proceeded via populated intermediates (36, 193, 219, 220). The observations completed a circle, however, as studies of small proteins were found to fold to the native conformation without obligatory intermediates (3, 25). Concomitantly, the role of folding intermediates became increasingly controversial (6, 8).
The evolution of experimental observations has been paralleled by the development of new models to describe folding reactions. The prevailing view a decade ago was that a pathway, a specific sequence of events narrowing the conformational search space, guided the folding reaction of proteins (176, 219, 221). Computational studies on simple lattice models, however, emphasized that folding can proceed along multiple pathways and that a funnel picture, consistent with the finding of kinetically two-state proteins, was more appropriate (32, 33). However, the emerging picture from more recent lattice models (34), all-atom molecular dynamics calculations (186, 212), and mutational studies (105, 209) is that a pathway, a bias in the energy landscape toward particular substructures, is more plausible. In tracing the pathways to “pathways” (186) circle, increased appreciation of the ensemble nature of the participating states and a stronger bridge between experiment and theory has been obtained.

B. Opportunities for Insights Into the Barriers in Folding Reactions

Studies on the nanosecond to millisecond time-scale using ultrafast mixing, temperature jumps, and pressure jumps will address questions on the fundamental events in the folding of proteins, extending work on model peptides. The consistency in the time-scales observed for rapid folding events, for example, in cytochrome c, apomyoglobin, monomeric λ repressor, and in a model β-hairpin suggest common underlying events that control early folding reactions. Complementary structural information on polypeptides, such as distance distributions, may be obtained by combining these ultrafast methods with excitation energy transfer studies (222–224) and small-angle X-ray scattering (19, 225).

Single-molecule spectroscopy, in which various fluorescence properties such as excitation energy transfer and polarization anisotropy can be monitored, are likely to lead to significant advances in our understanding of the folding reaction at a molecular level and in visualization of the dynamics along the folding barrier (226, 227). These studies will complement those in which the contributing factors to friction along the reaction coordinate are addressed. Single-molecule spectroscopy has been previously suggested as a means for observing the trapping dynamics predicted in analytical models of protein folding (147).

Development of algorithms for the prediction of stability of β-sheet structures will be very useful in applications of diffusion-collision type calculations to nonhelical proteins. This is a more formidable problem than that for α-helices owing to significant contributions from nonlocal interactions.
The field of protein folding reactions at elevated pressure is rapidly emerging. Further studies of the pressure dependence of refolding/unfolding rates will provide essential insights into the nature of barrier dynamics.

Recent MD simulations using massively parallel computers have provided an unprecedented view of a folding reaction. The simulation (124), which extended to nearly 1 μs, brings computational studies to an experimentally accessible time window for folding studies.

As is made abundantly clear throughout this review, the merging of computational and experimental studies offers the prospect of unprecedented insight into the structure and dynamics of barriers in protein folding reactions.

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REFERENCES

20. Jacob, M., Holtermann, G., Perl, D., Reinstein, J., Schindler, T., Geeves, M. A., and
28. van Nuland, N. A. J., Chiti, F., Taddei, N., Raugei, G., Ramponi, G., and Dobson,
   38, 1018–1029.
   Acad. Sci. USA 95, 11037–11038.
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