

Theoretical predictions of folding pathways by using the proximity rule, with applications to bovine pancreatic trypsin inhibitor

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Communicated by Robert Zwanzig, National Institutes of Health, Bethesda, MD, October 24, 1994

ABSTRACT We propose a phenomenological theory that accounts for entropic effects due to loop formation to predict pathways in the kinetics of protein folding. The theory, the basis of which lies in multiple folding pathways and a three-stage kinetics, qualitatively reproduces most of the kinetic measurements in the refolding of bovine pancreatic trypsin inhibitor. The resulting pathways show that nonnative kinetic transients are involved in the productive routes leading to the formation of native intermediates. Our theory emphasizes the importance of the random origin of chain folding initiation structures in directing protein folding.

Several *in vitro* experiments have established that many proteins can be made to fold spontaneously from an unfolded configuration. However, the determination of the folding mechanism has remained elusive largely because of the transient nature of intermediates. The only protein for which a detailed folding mechanism has been postulated is bovine pancreatic trypsin inhibitor (BPTI). In the mid-seventies Creighton (1, 2) devised ingenious methods to trap the disulfide-bonded intermediates, and he described folding pathways in terms of the intermediates that accumulate substantially during the folding process. Native BPTI contains three disulfide bonds; we denote the native species as [30-51; 5-55; 14-38], which indicates that Cys³⁰ is bonded to Cys⁵¹ and so on. There are 75 possible intermediates containing one or more disulfide bonds in BPTI. Of these, only 8 accumulate to detectable levels when examined on the time scale of the experiments (1–3).

The most surprising discovery in these studies is that three nonnative states, namely intermediates with disulfide bonds not present in the native state, are well populated. Two of the nonnative species, [30-51; 5-14] and [30-51; 5-38], are involved in the productive pathways—i.e., folding proceeds through either of these two kinetically equivalent intermediates (1–3). These findings had been well accepted until recently, when Weissman and Kim (WK) (4, 5) challenged Creighton's findings after reexamining the folding of BPTI (6, 7). Using different quench techniques to trap the intermediates, WK have found productive pathways in BPTI which are in apparent conflict with the earlier studies. The most glaring difference is that in the productive pathway only native intermediates play a significant role. Nonnative species may only be involved as required by disulfide chemistry (5) in the last stages of folding of BPTI—i.e., they may play a role in the formation of the precursor [30-51; 5-55] from [30-51; 14-38] (denoted by N^{sh} and N', respectively).

In this paper, we introduce a simple phenomenological rule, referred to as the *proximity rule*, to predict folding pathways in globular proteins. The underlying theoretical approach is quite general. Here, we apply the theory to analyze the folding pathways in BPTI. The rule yields a distinct folding mechanism

and qualitatively reproduces most of the experimental observations on the refolding kinetics of BPTI. The most important prediction of our theory is that the nonnative transient [30-51; 5-14] is a kinetically preferred state in pathways leading to the formation of native two-disulfide intermediates prior to the rate-limiting step. The latter step involves the rearrangement of N' to N^{sh} via nonnative kinetic states, most notably [30-51; 5-14].

Proximity Rule

Folding in disulfide-bonded globular proteins, such as BPTI and ribonuclease A, is believed to occur by disulfide bond rearrangement (1, 2). Thus, the conformations of intermediates needed for describing the folding pathways are specified by the positions of the Cys residues involved in the disulfide bond formation. This observation allows us to propose the proximity rule based on two general principles. (i) *Loop formation probability*. We assume that the intramolecular disulfide bond formation is a random process whose probability of occurring depends only on the loop length $l = |i - j|$, where i and j are the positions of the Cys residues along the polypeptide chain. The probability distribution for loop formation $\mathcal{P}(l)$, which is obtained from statistical mechanical arguments, is shown in Fig. 1. For simplicity we further assume that the probability for simultaneous formation of loops of sizes l_1 and l_2 , $\mathcal{P}(l_1, l_2)$, is proportional to $\mathcal{P}(l_1)\mathcal{P}(l_2)$. (ii) *Folding kinetics*. Following explicit studies of the kinetics of approach to the native state from a denatured state in lattice model of proteins (8), as well as estimates of the space of conformations in heteropolymers (9), we have concluded that folding occurs in three distinct stages. These stages are characterized by the following: I, a rapid collapse to a compact conformation; II, acquisition of native-like structure; and III, "all-or-none" transitions between metastable native-like states and the native state.

The probability distribution function $\mathcal{P}(l)$ must satisfy two requirements: first, $\mathcal{P}(l)$ should vanish when l is sufficiently small; second, $\mathcal{P}(l)$ should decrease for large enough l . If we take into account the approximate size of the residues, bond angle and dihedral angle constraints, and the covalent S—S bond length, then an estimate of the minimum value of l for establishing a disulfide bond between two Cys residues turns out to be around 7. Thus $\mathcal{P}(l) \approx 0$ for $l < 7$. In the fully denatured state, we view the protein as an extended polypeptide chain. In such a conformation, excluded-volume interactions between nonbonded residues dominate over weaker hydrophobic interactions. Hence, on the basis of purely entropic considerations, we expect the asymptotic functional form of $\mathcal{P}(l)$ to be similar to that found in homopolymers (10, 11), namely,

Abbreviations: BPTI, bovine pancreatic trypsin inhibitor; WK, Weissman and Kim.

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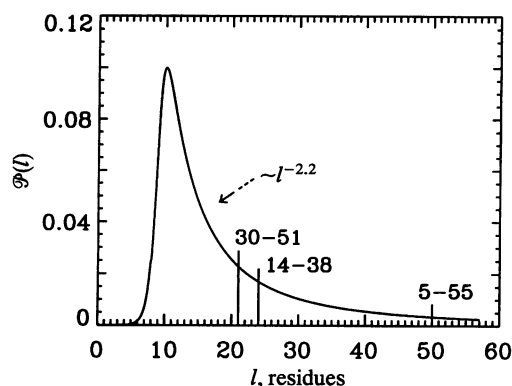


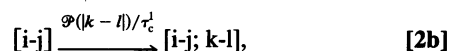
FIG. 1. Loop formation probability— $\mathcal{P}(l) = l^{-2.2} 18.42 / (\exp[1.8(9.0 - l)] + 1)$ —in a polypeptide chain as a function of loop length $l = |i - j|$, where i and j are the positions of two residues along the chain. The probabilities of forming the native disulfide bonds of BPTI are also indicated.

$$\mathcal{P}(l) \sim l^{-\nu(d+\theta_2)}, \quad [1]$$

where ν is the correlation length exponent and θ_2 is the critical index corresponding to interior loops [away from the ends of the chain (10)]—this yields $\nu(d + \theta_2) \approx 2.2$ for the space dimension $d = 3$. For loop sizes on the order of the size of the chain, $\mathcal{P}(l)$ deviates from Eq. 1, reaching a more or less constant value (see, e.g., ref. 12). For simplicity, we have neglected this finite-size effect. Our ansatz for $\mathcal{P}(l)$ is in good agreement with recent experiments of Darby and Creighton (13) in which the rates of formation of some of the single disulfides in BPTI were measured. The restrictions on $\mathcal{P}(l)$ at small and large values of l are satisfied by the representation shown in Fig. 1, in which we observe a peak at $l \approx 10$. The predictions of the proximity rule are to some extent independent of the details of $\mathcal{P}(l)$.

The three-stage kinetic scheme (8, 9) and $\mathcal{P}(l)$ allow us to define the proximity rule as follows.

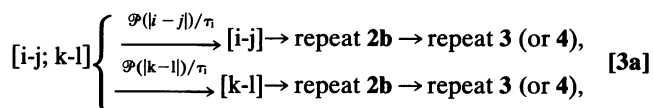
I. Random collapse. For short times, say τ_c^0 , the initial compaction of the protein takes place by *random* formation of single disulfide species. The concentration of the various single-disulfide species is determined by the probability distribution $\mathcal{P}(l)$. These early-forming chain-folding initiation structures (14) *direct* subsequent folding events. On a time scale $\tau_c^1 (\approx \tau_c^0)$, the initial single-disulfide intermediates form a second disulfide bond with relative populations determined by $\mathcal{P}(l)$. A schematic representation of this two-step process is given by



where R denotes the reduced protein, $[i-j]$ represents all possible pairs of Cys residues, and $[k-l]$ corresponds to all possible second pairs of Cys residues, and at each step the probabilities $\mathcal{P}(l)$ are normalized to unity.

II. Kinetic ordering. In this regime, the unit of time $\tau_i (\gg \tau_c^0)$ corresponds to the time needed for the breakup of the transient nonnative disulfide. On this intermediate time scale, nonnative two-disulfide and seminitative species interconvert to native species according to the following rules.

Nonnative case:



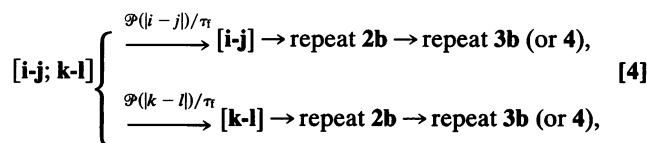
with $\mathcal{P}(|i-j|) + \mathcal{P}(|k-l|) = 1$.

Seminitative case: Let $[i-j]$ be a native disulfide bond,



As this process evolves the concentration of native-like intermediates increases at the expense of nonnative intermediates. This kinetic ordering is basically complete when most of the intermediate species are in native-like states.

III. All-or-none. The last stage of folding corresponds to transitions between native-like two-disulfide species and the native state. These transitions are mostly determined by the highest energy barriers, and then they occur on time scales $\tau_f \gg \tau_i$. Following Eq. 3a, the rearrangement of *native* disulfide bonds proceeds as



with $\mathcal{P}(|i-j|) + \mathcal{P}(|k-l|) = 1$.

It is not possible to predict the precursor to the native state by using the proximity rule alone. This would require assumptions about the free energies associated with the rearrangement of native-like two-disulfide species. Nevertheless, geometrical aspects of the native structure can often be used to determine the precursor. For example, in the case of BPTI, it is clear that the formation of native bonds [30-51] and [5-55], which are buried in the hydrophobic core, should be the rate-limiting step of the folding process (1, 4).

It should be stressed that the time constants τ_c , τ_i , and τ_f should be strongly dependent on noncovalent forces and solvent conditions such as pH and concentration of denaturants. Our theory does not provide independent expressions for the dependence of these time constants on the nature of solvents. In fact, we use these time scales as the only free parameters of the theory. Although two-disulfide intermediates are rearranged by breaking one disulfide bond and forming another one, the lifetime of the transient single-disulfide intermediates does not play an explicit role in the formulation of the kinetic rule as long as $\tau_c \ll \tau_i$. Therefore, whether intramolecular disulfide rearrangements occur with or without single-disulfide intermediates, the predictions of the proximity rule for the late stages of folding remain unchanged. Partly and fully folded conformations are implicitly taken into account by associating greater stability to native-like intermediates (15). Once the time scales are set the rearrangement rates in 2, 3, and 4 are fully determined. These rates, however, are used not as the coefficients of coupled differential equations but as the proportion of a given species that rearranges to its product at each unit of time. Thus, the kinetic scheme is solved in discrete time steps.

Folding Pathways of BPTI

Given the relative position of the Cys residues in the primary sequence of BPTI (5, 14, 30, 38, 51, and 55) and the disulfide bonds present in its native state ([30-51], [14-38], and [5-55]), we apply the proximity rule to predict the refolding kinetic pathways in BPTI. To assess the validity of the theory we have used the proximity rule to predict the rearrangement kinetics of the nonnative intermediates [30-51; 5-14] and [30-51; 5-38]. The predictions can be *directly* compared with WK experiments on the rearrangement kinetics of purified forms of these nonnative species (5).

Starting from a population of (i) only [30-51; 5-14] and (ii) only [30-51; 5-38], we have followed the rearrangement of all by-products by using rule 3b. To compare with the experiments, we have chosen the time scales for both cases such that the kinetic step (2b) is very fast, $\tau_c^1 = 1$; no rearrangement of native disulfides is allowed ($\tau_f = \infty$); and the overall time scale

is $\tau_i = 40$ sec. Notice that in this case τ_i is the only free parameter of the theory, and its value is chosen such that the experimental and theoretical time scales coincide. Fig. 2 shows that the theoretical predictions for the *entire* time evolution of the rearrangement of [30-51; 5-14] (Fig. 2A) and [30-51; 5-38] (Fig. 2B) are in excellent agreement with the experimental data. After 15 min, WK experiments indicated that both seminaive intermediates rearrange to [30-51; 14-38], [30-51; 5-55], and [14-38; 5-55] in amounts of approximately 80%, 10%, and 10%, respectively. These states are denoted by N' , N_{sh}^{sh} , and N^* , respectively. Our theory agrees well with these results, predicting a population of 80% N' and 16% N_{sh}^{sh} . According to the proximity rule N^* is not produced in this regime unless $\tau_i < \infty$ in 4. Our prediction for the formation of [30-51; 5-38] shown in Fig. 2A does not compare well with the experimental data. This difference suggests that we are probably underestimating the relative importance of [5-38] with respect to [5-14]. The state [30-51; 14-55] is also found in small amounts. The discussion concerning the formation of [30-51; 38-55] is given below.

Given the success of our comparison with experiments, we discuss the refolding of reduced BPTI. The random collapse regime in BPTI yields a distribution of one-disulfide species followed by two-disulfide transients, most of which are nonnatives. The relevant time scale in the kinetic ordering regime τ_i is such that $\tau_c^0 \leq \tau_c^1 \ll \tau_i \ll \tau_f (= 1000\tau_i)$. The time τ_i was chosen so that regime III becomes important only after regime II has already led to concentrations of only native-like intermediates. Fig. 3 shows the folding kinetics for Regime II and III as a function of time (in units of τ_i). Notice that the proximity rule predicts that the kinetic ordering regime should last for about two orders of magnitude, $(1-100)\tau_i$, which seems reasonable when compared with the time scales of WK experiments.

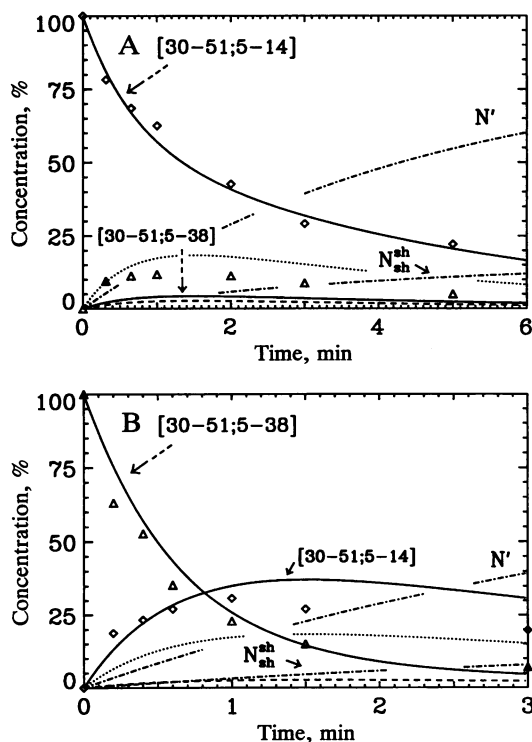


FIG. 2. Analysis of the rearrangement kinetics of [30-51; 5-14] (A) and [30-51; 5-38] (B) (see text). Curves represent the proximity rule predictions. Superimposed are the experimental concentrations of [30-51; 5-14] ($\diamond$) and [30-51; 5-38] (Δ) taken from ref. 5. Dotted and dashed lines correspond to [30-51; 38-55] and [30-51; 14-55], respectively.

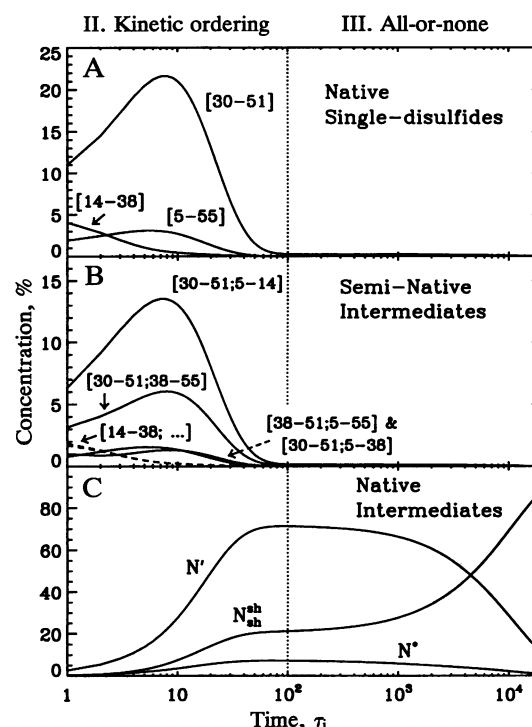


FIG. 3. Predictions of the proximity rule for the concentration of native single-disulfides (A), seminaive intermediates (B), and native intermediates (C) as a function of time (in units of τ_i) for the late stages of folding. The dotted line is a guide to the eye that indicates the approximate time separation of regimes II and III. In B [14-38; ...] indicates both [14-38; 5-30] and [14-38; 30-55]. Notice that the sum of A and C gives the full amount of conformations with native disulfides; everything else corresponds to nonnative states.

We first consider the production of single-disulfide species. According to our kinetic scheme, single-disulfide species form in the random collapse regime. A direct consequence of $\mathcal{P}(I)$ is that only a few of these species are native (see Fig. 3 at $\tau_i = 1$). Moreover, *native* single-disulfide species form—at least transiently—in the early stages of the kinetic ordering regime as a result of the rapid interconversion of seminaive two-disulfide species. For example, initially the largest concentration of single-disulfide species is nonnative [5-14]; in all likelihood [5-14] rearranges further to eventually form [5-14; 30-51], which in turn leads to the production of native-like [30-51]. Although this mechanism is not as direct as the one favored in the literature (see, e.g., refs. 1–7), it is the *simplest* theoretical assumption consistent with Darby and Creighton (13). Indeed, if fast folding experiments unambiguously establish that native single-disulfide species are formed directly from R, then the basic kinetic scheme given here should be altered to allow for the reverse reaction in 2a to occur faster than the forward reaction in 2b. This, of course, would add an extra free parameter to the theory. However, inasmuch as experiments are not conclusive regarding this issue we have tried to keep the formal theory as simple as possible.

Fig. 3A shows the concentration of these native single-disulfides as a function of time. Although $\mathcal{P}(I)$ predicts that the probability of formation of [14-38] is considerably greater than that of [5-55], we find that in the kinetic regime (II) the concentration of [14-38] decreases rapidly, while the concentrations of [30-51] and [5-55] increase. This is one of the most interesting predictions of the proximity rule. The reason for this difference is that once [14-38] is formed, $\mathcal{P}(I)$ predicts that the most probable two-disulfide intermediate is [14-38; 30-51], which, being a native intermediate, does not undergo further rearrangement on the time scale of the kinetic ordering regime (this possibility was already hinted at by WK in ref. 4). On the

other hand, the most probable double-disulfide species formed from [30-51] and [5-55] are [30-51; 5-14] and [5-55; 38-51], respectively. These transient intermediates dissociate again and hence the overall concentration of [30-51] and [5-55] increases for intermediate times.

Fig. 3A offers a very striking comparison with experiments. At $t \approx 8\tau_i$ our theory predicts that the ratio of the maximum concentration of [30-51] to that of [5-55] is 7:1 and the concentration of [14-38] is negligible. This ratio is in excellent agreement with the experiments of WK, who find a ratio (4) of 6:1, and is in disagreement with Creighton's estimate of 20:1 (2). These comparisons imply that experiments have not been able to measure the early fast folding events of the random collapse, but in all likelihood they are probing the rearrangements that occur in the kinetic ordering regime. This assertion is consistent with the fact that experiments (1, 2, 4) are performed on a time scale of minutes, whereas the value for τ_i extracted from Fig. 2 is less than minutes.

The rearrangement kinetics of seminate intermediates, shown in Fig. 3B, indicates that only nonnative [30-51; 5-14] accumulates to significant concentrations during the early stages of the kinetic ordering regime. Because single disulfides are mostly produced by the rearrangements of seminate states, the concentrations of two-disulfide intermediates follow very closely those of single-disulfide species. Accordingly, nonnative intermediates involving [14-38], like [14-38; 5-30] and [14-38; 30-55] (see Fig. 3B), are depleted early in the folding process. One drawback of the theory is that nonnative intermediate [30-51; 38-55] is more populated than [30-51; 5-38]. This seems to be a consequence of the simplification regarding the two-loop formation probability. It is known that this assumption is not strictly valid (17, 18). Although a more refined version of our theory that would prohibit the formation of the tight cross-linking of residues can be formulated, the necessary addition of extra free parameters discouraged us from doing so. By blocking the formation of [30-51; 38-55], however, we have checked that this transient is not essential for any of our conclusions.

The kinetics of formation of the native two-disulfide intermediates N' , N_{sh}^{sh} , and N^* are shown in Fig. 3C. The production of these native states increases sharply only after the concentration of single-disulfide species, largely [30-51] and [5-55], begins to decrease rapidly (see Fig. 3A and B). We find that at the end of the kinetic ordering regime ($t \approx 10^2\tau_i$), N' is the most populated intermediate, with a concentration of 71%. This is in agreement with WK's experiments. The state N^* , which originates from nonnative species involving [5-55], is found in small amounts on the order of 7%. The precursor to the native state N_{sh}^{sh} accumulates to roughly 21%. Although this amount of N_{sh}^{sh} may seem rather high, the prediction that a small proportion of N_{sh}^{sh} is formed by the same mechanism as

N' is in accord with experiments on the rearrangement kinetics of purified forms of [30-51; 5-14]. Our theory predicts that 13% of these pathways originate from [30-51; 5-14] and other nonnative intermediates involving [30-51], whereas 8% come from nonnatives involving [5-55]. It should be emphasized, however, that these transient species accumulate to significant levels during the early stages ($t \approx 10\tau_i$ in Fig. 3B) of the kinetic rearranging regime. We note that N_{sh}^{sh} is reached through pathways that involve almost exclusively nonnative intermediates. These findings imply that even in the absence of Cys¹⁴ or Cys³⁸ nonnative alternative pathways leading to the formation of N_{sh}^{sh} can be found. This is consistent with refolding experiments using modified forms of BPTI (1, 2, 19, 20). The calculations based on the proximity rule show that almost all native intermediates (including N') originate from nonnative transients, most notably [30-51; 5-14] (see Fig. 4).

As previously discussed, our theory for the all-or-none regime (III) ($t \approx 10^2\tau_i$ in Fig. 3) should be regarded with caution. In this time regime, native two-disulfide intermediates N' and N^* rearrange according to 4; the precursor N_{sh}^{sh} is assumed not to undergo further rearrangements. We find that the state N^* rearranges almost exclusively to N' . The proximity rule indicates that the native state is *primarily* obtained by the rearrangement of the two-disulfide species N' . The rearrangement kinetics of N' is such that 90% of the transitions to the precursor N_{sh}^{sh} occur via nonnative kinetic states.

Discussion

The most controversial aspect of the refolding experiments of BPTI is in the assessment of the role of nonnative intermediates [30-51; 5-14] and [30-51; 5-38] in the preferred folding pathways. We find that the kinetic state [30-51; 5-14] is a preferred intermediate involved in almost all pathways leading to the formation of N' . The important role played by this nonnative state has been further confirmed by studying the pathways in modified forms of BPTI in which we *blocked* the formation of all possible seminate intermediates one at a time (15 in total). We found that *only* when the formation of [30-51; 5-14] was inhibited did the rates of formation of most states vary significantly. Furthermore, the overall kinetics is slowed by a factor of 4. Fig. 3 shows that in the late stages of folding nonnative species should be difficult to detect in refolding experiments of normal BPTI. Nonetheless, the theoretical prediction is that [30-51; 5-14] is the predominant intermediate in the rearrangement of N' to N_{sh}^{sh} . In fact, [30-51; 5-14] may be the transition state in the rate-limiting step. In contrast to the role played by [30-51; 5-14], the proximity rule suggests that [30-51; 5-38] is not essential for folding. Indeed, when formation of [30-51; 5-38] is blocked, alternative pathways are found such that the overall kinetics remains intact.

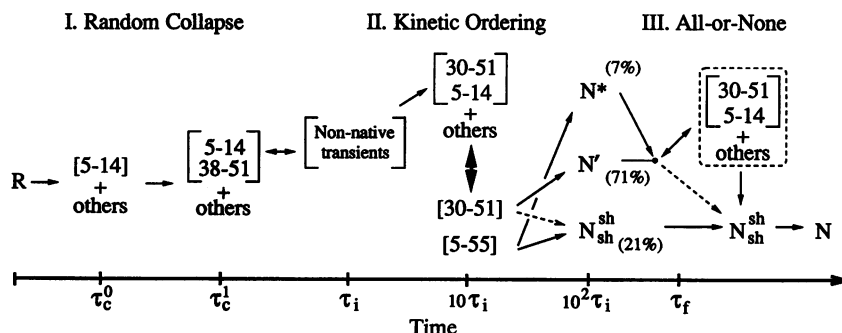


FIG. 4. Sketch of the most productive pathways in the folding of BPTI as a function of an approximate logarithmic time scale, with $\tau_c \ll \tau_i$ means that other states of the same nature as [A] are possible, but less likely. We note that in regime I "others" involve a particularly broad distribution of states—e.g., [38-51], [14-30], etc. Dashed arrows correspond to transitions with smaller rate constants than solid arrows. The dashed box represents kinetic states whose lifetimes are much smaller than those of other species present in that time regime.

Our conclusions are summarized in Fig. 4, where the productive pathways of native BPTI are sketched. This folding scheme underscores the importance of the random origin of the chain folding initiation structures, and the loop formation probability $\mathcal{P}(l)$. When combined with the additional knowledge of the Cys residues paired in the native state, these two elements yield the essential step of our kinetic scheme from which the *nonsequential* nature of the folding process and the majority of the conclusions follow.

One caveat in our theory is that the important roles played by noncovalent forces and precise solvent conditions in folding are almost ignored. It is likely that these factors could be responsible for the greater stability that we have assigned to native-like bonds (τ_f) with respect to nonnative bonds (τ_i). Nonetheless, at present the theory does not provide independent theoretical estimates for the time scales τ_i and τ_f . For BPTI, the value of $\tau_f = 1000\tau_i$ was chosen to be the longest time scale in the problem. The latter seems to be consistent with the observation that the precursor N_{sh}^{sh} is mostly formed at very late times. We should note that recently Zhang and Goldenberg (21) have found that the overall rate of formation of N_{sh}^{sh} could be substantially increased by replacing Tyr at position 35 by Leu in BPTI. We assume that this observation corresponds to $\tau_f/\tau_i \approx 1$. If we use this time ratio, leaving everything else exactly the same as for the wild-type protein, we find that the theoretical kinetic curves analogous to Fig. 3 reproduce several aspects of the refolding experiments of Y35L BPTI (unpublished results). For example, the N_{sh}^{sh} production is enhanced considerably at the expense of N' , the concentration of [30-51] is increased, whereas that of [5-55] is nearly eliminated. Hence, using the ratio of τ_f/τ_i as an adjustable parameter, and to the extent that these parameters are realistic, we believe that our current theory may be helpful in analyzing kinetic experiments.

The major limitation of our theory is that we have not explicitly included the role of noncovalent interactions. Furthermore, our assumptions are reasonable only in an *average* sense, implying that most of the intrinsic specificities of each protein are ignored. Nonetheless, developing this theory for a generic disulfide-bonded globular protein has allowed us to test the generality of a different theoretical approach to folding. As far as we are aware, we have provided for the first time a theory that centers on the intertwined roles that entropy and kinetics may have in the folding process. Inasmuch as some striking predictions of the theory are in good agreement with experiments (1, 2, 4, 5, 13), we believe that our approach has succeeded in shedding some light on this complex problem.

Recent experiments (22) on optically triggered folding of cytochrome *c* have suggested that the proximal formation of loops as an initiating event in protein folding could be general. The time scale for the formation of loops was estimated to be about 0.1 msec, which is in good agreement with the theoretical

predictions (23). However, many questions still remain unanswered by the present theory. We have suggested that solvent conditions and noncovalent forces should affect the folding time scales, but how does this occur? How can we include the effects of secondary structure constraints and compactness on the kinetics and $\mathcal{P}(l)$? In principle, the success of $\mathcal{P}(l)$ appears to validate a folding mechanism that proceeds by unfolding-folding as described by the kinetic equations. We should also mention that simulations of minimal protein folding models indicate that rearrangement of intermediates involves considerable unraveling of the protein (8). To address these and other important questions specific interactions responsible for driving the folding process need to be explicitly considered.

We are grateful to Dr. T. E. Creighton for a careful reading of the manuscript and for bringing some relevant references to our attention. This work was supported in part by a grant from the National Science Foundation. C.J.C. acknowledges support from the Fondo Nacional de Ciencia y Tecnología (Chile).

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