The Disulfide Folding Pathway of BPTI

In their Research Article "Reexamination of the folding of BPTI: Predominance of native intermediates," J. S. Weissman and P. S. Kim (*1*) state that the disulfide pathway of unfolding and refolding of the protein BPTI (bovine pancreatic trypsin inhibitor) (*2*, *3*) (Fig. 1) is incorrect and in need of revision. However, the data presented by Weissman and Kim are fully consistent with the fundamental aspects of the original pathway and indeed confirm it; in contrast, their revised pathway is inconsistent in several respects with the available experimental data.

Weissman and Kim have reinvestigated the BPTI folding pathway, using reversed-phase high-performance liquid chromatography separations to characterize the disulfide intermediates that are trapped by blocking all Cys thiols during refolding and disulfide formation from the reduced protein. Their separation procedures are clearly superior to those used in earlier work and represent a substantial advance. Using these procedures, Weissman and Kim confirmed many of the aspects of the original disulfide folding pathway. In particular, they found the same trapped one-disulfide intermediates that were detected originally (*4*, *5*) and confirmed that by disulfide "shuffling" these intermediates are in a rapid equilibrium in which [30-51], with a disulfide bond between Cys^{30} and Cys^{51}, predominates. The same two-disulfide intermediates were identified as had been observed previously (*2*, *3*), and the rearrangements they undergo in the folding process (*2*) were confirmed.

Two observations by Weissman and Kim are new and pertinent. The first is that the use of the previous diagonal mapping methods (*4*) may have resulted in an overestimation of the amount of the second most predominant single-disulfide intermediate [5-30], which has a nonnative disulfide. The second is that the nonnative two-disulfide intermediates [30-51; 5-14] and [30-51; 5-38], which will be designated collectively here as [30-51; 5-14/38], were trapped by acid in substantially lower quantities than they were when alkylated with iodoacetamide or iodoacetate. Both of these observations are plausible, but the original kinetic analysis of the BPTI pathway was not dependent on the actual amounts of these individual intermediates. Consequently, the observations of Weissman and Kim do not invalidate the original BPTI folding pathway.

The analysis of Weissman and Kim mistakenly equates the amount to which an intermediate accumulates with its kinetic importance. For example, with sequential pathways where intermediates accumulate for solely kinetic reasons, those intermediates that accumulate the most are those with the slowest rate of further progression and, hence, may be the least productive. Thus, a revised BPTI folding pathway that omits the intermediates with nonnative disulfides, because they did not accumulate in large amounts, is not warranted by the data presented by Weissman and Kim.

Many of the disulfide intermediates in BPTI folding are in rapid equilibrium during folding, and consequently their kinetics of appearance and disappearance are the same. The kinetic roles of these intermediates have been determined by the kinetic effects of removing the Cys thiols, either by site-directed mutagenesis (6–9) or by blocking them irreversibly (*2*). The refolding properties of several such modified proteins (*2*, *6*) were the basis for certain aspects of the original pathway (*2*, *3*). These data were not considered by Weissman and Kim, and they are inconsistent with their proposed pathway.

The revised pathway of Weissman and Kim has [30-51; 14-38] and [5-55; 14-38] as obligatory intermediates in refolding, but this is incompatible with the folding properties of BPTI with either one or the other of the Cys^{14} or Cys^{38} thiols blocked irreversibly (*2*). Neither of these proteins can form the 14-38 disulfide bond and, according to the revised pathway, disulfide bond formation should essentially stop at the one-disulfide stage. Both of these proteins have been shown (*2*), however, to refold to native-like [30-51; 5-55] at intramolecular rates that collectively are one-third of the normal rate (*3*). The substantial rates of refolding of these modified proteins were the basis for including in the original pathway the formation of intermediates [30-51; 5-14/38] directly from the one-disulfide intermediates (Fig. 1). Reduced analogs of the [30-51; 5-14/38] intermediates have since been observed to form the two disulfide bonds at the expected rates (*9*). Removing or blocking both Cys^{14} and Cys^{38} slows, by a factor of 10^{-5}, the intramolecular steps both in refolding of the reduced protein to [30-51; 5-55] and in its unfolding and disulfide reduction (*2*, *6*, *7*). These data show that intermediates with nonnative disulfides, such as [30-51; 5-14/38], are the kinetically important two-disulfide intermediates and that [30-51; 14-38] and [5-55; 14-38] are not; opposite roles are attributed to them in the revised pathway of Weissman and Kim.

Weissman and Kim assert that the disulfide folding of BPTI is much more relevant at neutral pH than at the usual pH of 8.7 because the accumulation of native-like conformations is much greater at a lower

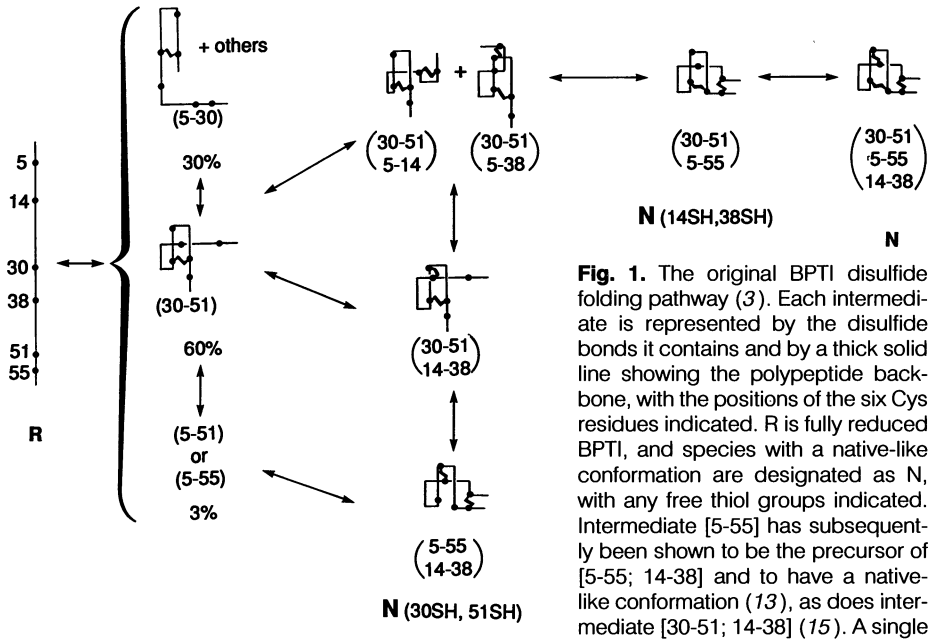

Fig. 1. The original BPTI disulfide folding pathway (*3*). Each intermediate is represented by the disulfide bonds it contains and by a thick solid line showing the polypeptide backbone, with the positions of the six Cys residues indicated. R is fully reduced BPTI, and species with a native-like conformation are designated as N, with any free thiol groups indicated. Intermediate [5-55] has subsequently been shown to be the precursor of [5-55; 14-38] and to have a native-like conformation (*13*), as does intermediate [30-51; 14-38] (*15*). A single kinetic step and a bracket represent formation of all the single-disulfide intermediates because of their rapid intramolecular rearrangement with each other. The major one-disulfide species are indicated as originally estimated, but the new data (*1*) indicate that the amount of [5-30] may have been overestimated. The "+" between intermediates [30-51; 5-14] and [30-51; 5-38] indicates comparable kinetic roles. The identity of the intermediate in rearrangement between [30-51; 14-38] and [5-55; 14-38] is not known, but the sulfur atoms of Cys^{30}, Cys^{51}, Cys^{5}, and Cys^{55} are in close proximity in the native conformation. [Reprinted with relettering with permission from (*3*)]

pH, where the protein thiol groups are not ionized. But these are just the conditions where the disulfide approach to folding is least appropriate. The use of disulfides to elucidate protein folding mechanisms is possible only when the reduced protein is unfolded, so that disulfide bond formation and protein folding are linked functions (*10*). Ionization of thiol groups at alkaline pH contributes to keeping a reduced protein unfolded. At lower pH, protonated thiol groups are more readily buried by the protein adopting the native conformation. This is a major factor with an exceptionally stable folded protein like BPTI. Molecules of this protein with two native-like disulfide bonds, and even the molecule with only the most stable disulfides 5-55, tend to adopt a native-like conformation (*8, 11–15*). The stability of the native conformation is lowered, but it can be populated even at pH 8.7 with (30-51; 14-38) and (5-55; 14-38). The native-like conformation with buried thiols is more stable at lower pH, and this is undoubtedly the reason why native-like species tend to predominate under these conditions (*1*). The occurrence of fully folded conformations is of little relevance to the process of folding. Disulfide bond formation in such quasi-native species is governed primarily by thiol group accessibility and reactivity and is not linked to folding. The pathway of disulfide bond formation in BPTI that is most relevant to folding is that at higher pH by way of the one-disulfide intermediates, especially [30-51], the nonnative two-disulfide intermediates [30-51; 5-14/38], and the native-like [30-51; 5-55].

In their revised pathway, Weissman and Kim depict the formation of the 14-38 disulfide bond in intermediate [30-51] as being "very fast," the rate specified for the native-like intermediate [30-51; 5-55]. This implies that intermediate [30-51] has a very native-like conformation, which is implied by Weissman and Kim solely on the basis of a partial peptide model (*16*). Yet all the kinetic data available (*2, 3*), obtained under the usual conditions of pH 8.7, indicate that [30-51] forms second disulfides during refolding at no more than 0.005 the rate that native-like [30-51; 5-55] forms the 14-38 disulfide. This slow rate of formation of the 14-38 disulfide bond indicates that the [30-51] intermediate does not have a totally native-like conformation during folding under the conditions normally used. This has been confirmed by nuclear magnetic resonance analysis of the trapped intermediate (*12*) and of an analog in which the four other Cys residues were replaced by Ser (*17*). Intermediate [30-51] has some elements of the native conformation, as suggested by the peptide model (*16*), but the remainder of the polypeptide chain is disordered (*17*). The disordered portions include Cys^5, Cys^{14}, and Cys^{38}, which explains why they can readily form any of the three possible disulfide bonds between them. The partially folded conformation of [30-51] is not disrupted by formation of the nonnative 5-14 and 5-38 second disulfide bonds, and analogs of the [30-51; 5-14/38] intermediates have partially folded conformations that are similar to that of [30-51] (*18*). Contrary to the assertion of Weissman and Kim, the conformational properties of the [30-51] and [30-51; 5-14/38] intermediates are fully compatible with their original roles in folding.

Weissman and Kim imply that accessibility of thiol groups within the native-like conformation is a major factor specifying the BPTI pathway. This is undoubtedly the case with the quasi-native species [5-55] (*13*) and [5-55; 14-38] (*11, 14*), where the Cys^{30} and Cys^{51} thiols tend to be inaccessible and unreactive because of the native conformation. Inaccessibility of thiols is not the cause, however, of the relative blockages in disulfide formation with the other species in the original folding pathway, such as [30-51] ⇸ [30-51; 5-55] and [30-51; 14-38] ⇸ N (Fig. 1). The Cys^5 and Cys^{55} thiol groups of the [30-51] and [30-51; 14-38] intermediates are sufficiently reactive to disulfide reagents to accumulate as the mixed disulfide when they cannot readily form a protein disulfide (*2, 19*). This accumulation as the mixed disulfide is positive evidence that the block in forming these disulfide bonds occurs in the second, intramolecular step, in which occurs folding and the displacement by a second Cys thiol of the mixed disulfide, not because of inaccessibility of the thiol groups (*20*).

In summary, the original BPTI folding pathway (Fig. 1) is consistent with all the experimental data available and remains valid, in contrast to the revised pathway of Weissman and Kim.

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30 September 1991; accepted 4 December 1991

Response: Creighton accepts the accuracy of our folding experiments with bovine pancreatic trypsin inhibitor (BPTI), but he asserts that our analysis mistakenly equates levels of accumulation of intermediates with their kinetic importance. However, our kinetic analysis did not rely on the population of the observed intermediates. Rather, each of the rates indicated in our BPTI folding pathway (*1*) was measured directly with chromatographically purified, reversibly trapped intermediates (Fig. 1). Moreover, as we will explain below, our conclusions are fully consistent with the experimental data cited by Creighton.

Creighton's pioneering work introduced the trapping of disulfide-bonded intermediates as a method for studying the folding of proteins. This work also established the folding pathway of BPTI as perhaps the most important paradigm for protein folding. Two features of the original BPTI folding pathway (*2*) were particularly striking. First, there were specific nonnative species (that is, molecules containing disulfide bonds not found in the native protein) that were populated at high levels. Second, two of the well-populated nonnative species had a critical role in guiding BPTI to its native state. These fundamental aspects of the original folding pathway for BPTI provided the strongest available evidence that nonnative interactions play an important and specific informational role in protein folding.

In our paper (*1*), we reexamined the folding of BPTI with identical buffer conditions and pH (8.7) as in the original BPTI folding experiments. In this reexamination, we found that only intermediates with native disulfide bonds are well populated during folding. In addition, our kinetic studies led to the conclusion that the salient features of BPTI folding are determined primarily by native structure in these well-populated folding intermediates. More specifically, we concluded that structure in [30-51; 14-38], the native two-disulfide intermediate that precedes the rate-limiting step, by burying and constraining the free thiols of Cys^5 and Cys^{55}, was both preventing direct oxidation and inhibiting rearrangement of this intermediate. This con-

clusion was supported by two-dimensional nuclear magnetic resonance spectroscopy (2D-NMR) studies suggesting that [30-51; 14-38] was folded completely into a native conformation [see figure 7 of (*1*)]. Moreover, we found that the rearrangement of [30-51; 14-38] was accelerated substantially by the addition of six molar urea, indicating that this rate-limiting step required substantial unfolding of previously acquired native structure.

Creighton's criticisms of our paper (*1*) are based on his assertion that, in contrast to our conclusions, species with nonnative disulfides are the kinetically important folding intermediates. We will answer, in order, each of the specific issues raised by Creighton.

First, our BPTI folding pathway does not predict that folding should stop at the one-disulfide stage if either Cys^{14} or Cys^{38} is blocked. The kinetically preferred route (Fig. 1) for forming [30-51; 5-55], the immediate precursor to native BPTI, is [30-51] → [30-51; 14-38] → [30-51; 5-55]. This preferred route is prevented by blocking Cys^{14} or Cys^{38}. The protein is then free to fold by an alternate route. Indeed, such alternate routes can be unmasked by studies of modified BPTI molecules lacking particular thiols (*3–6*). Thus, [30-51; 14-38] and [5-55; 14-38] are not, as Creighton states, obligatory intermediates in our BPTI folding pathway, and this pathway is fully compatible with the observation that BPTI can fold to [30-51; 5-55] when the thiols at Cys^{14} or Cys^{38} are chemically modified.

Second, mutating both Cys^{14} and Cys^{38} does not retard the rate of forming [30-51; 5-55] by a factor of 10^5. In fact, Creighton's earlier folding studies (*3, 7*) indicated that the rate of forming [30-51; 5-55] in the Cys^{14}, Cys^{38} double mutant protein is the same as the rate of forming [30-51; 5-55] in the wild-type protein (*8*). This issue has been discussed lucidly by Goldenberg (*4*). Briefly, the two-disulfide species that are formed rapidly in the wild-type protein (denoted II in Fig. 2) do not include the [30-51; 5-55] intermediate (*3, 4*). The rate of forming [30-51; 5-55] from II in the wild-type protein is slow and very similar (*8*) to the rate of forming [30-51; 5-55] directly from the one-disulfide intermediates (denoted I) in the Cys^{14}, Cys^{38} double mutant protein (Fig. 2). As a consequence, the rates of folding to [30-51; 5-55] in the wild-type and the Cys^{14}, Cys^{38} double mutant proteins are almost identical (*4, 5, 8*). Thus, the folding properties of modified proteins actually provide strong evidence against the notion that nonnative disulfide species accelerate the folding of BPTI.

Third, the intramolecular step in the reduction of [30-51; 5-55] is not known to be 10^5 times slower in the Cys^{14}, Cys^{38} double mutant. Previously (*3, 4*), the rate of reduction of the mutant protein was measured with dithiothreitol (DTT) as the reducing agent and was found to be dependent on the concentration of DTT. These measurements therefore reflect intermolecular processes and cannot be used to infer the rate of the intramolecular steps in unfolding and disulfide reduction [for a review, see (*9*)].

Fourth, ionization of the thiol groups is not required to unfold reduced BPTI. Creighton (*10*) has concluded that reduced BPTI is grossly unfolded, both at neutral pH and at pH 8.7. Thus, protein folding and disulfide bond formation are linked functions for BPTI at both neutral and alkaline pH.

Fifth, our studies demonstrated that intermediates with native disulfide bonds predominate not only at neutral pH, but also at pH 8.7 (that is, in conditions identical to those used in the original BPTI folding studies). Moreover, our reason for advocating neutral pH for future studies of BPTI folding was not, as is asserted, to promote the accumulation of native-like conformations. As we stated in our paper (*1*), we studied folding at neutral pH so as to avoid working near the pK_a of the cysteine thiols. Because the pK_a value for cysteine thiols is close to 8.7, each cysteine residue will be deprotonated approximately half of the time at pH 8.7. Thus, a particular disulfide intermediate at pH 8.7 will actually consist of a heterogeneous population of molecules, with different ionization states at different cysteine residues. In addition, structure in intermediates can alter the pK_a of the cysteines. Therefore, results obtained at neutral pH should simplify interpretation of the structural basis of the folding pathway. (The stated goal for advocating alkaline pH, to destabilize the folding intermediates, could be accomplished effectively without introducing chemical heterogeneity into the folding intermediates, by adding denaturants, increasing the temperature, or introducing destabilizing point mutations into the protein.)

Sixth, the rate of oxidation of the 14-38 disulfide bond in the native intermediate [30-51] was not based on a peptide model. This rate and all other rates indicated in our folding pathway (Fig. 1) were determined directly, starting with a pure, reversibly trapped intermediate (*1*). Moreover, the

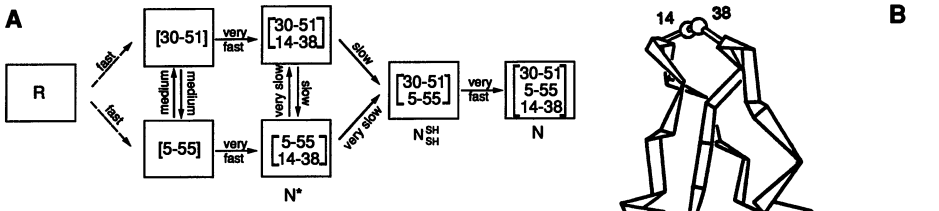

Fig. 1. (**A**) The kinetically preferred pathway for the folding of BPTI (*1*). R denotes the reduced protein, which is grossly unfolded both at neutral and alkaline pH (*10*). All the well-populated intermediates contain only native disulfide bonds. The relative rates of the intramolecular transitions associated with each step at pH 7.3, 25°C, are indicated. These rates were determined by purifying individual intermediates to homogeneity at low pH, and then, taking advantage of the reversibility of acid quenching, following the rearrangement and folding of these intermediates in isolation. N* is a kinetically trapped intermediate that is stable for months in these conditions. The dotted arrows indicate that R is oxidized initially to a broad distribution of one-disulfide intermediates, which then rearrange rapidly to [30-51] and [5-55]. (**B**) A diagram of native BPTI with its three disulfide bonds [adapted from (*13*)].

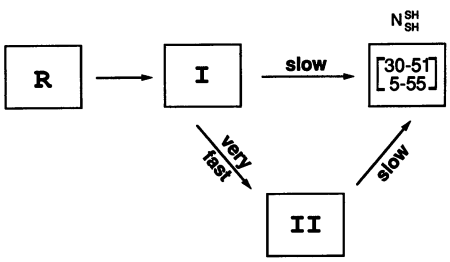

Fig. 2. Simplified scheme of the folding of reduced BPTI to [30-51; 5-55], the immediate precursor to native BPTI, also known as N^{SH}_{SH}. Qualitative estimates of the relative rates of the intramolecular transitions associated with each step are indicated (*1, 3, 4, 7, 8*). R denotes the reduced protein, I denotes collectively the one-disulfide intermediates, and II denotes the two-disulfide intermediates that are formed rapidly in the wild-type protein. The predominant component of II is the [30-51; 14-38] intermediate (*1*). If both Cys^{14} and Cys^{38} are blocked (*3*) or mutated (*4, 5*), then [30-51; 5-55] is formed by direct oxidation of the one-disulfide species (I). At pH 8.7, 25°C, the intramolecular step for direct oxidation (that is, I → [30-51; 5-55], measured in the mutant protein) occurs at approximately the same rate (*8*) as the intramolecular step for rearrangement (that is, II → [30-51; 5-55], measured in the wild-type protein).

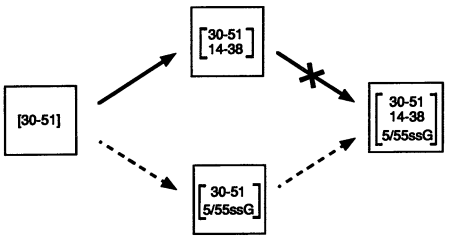

Fig. 3. Two possible routes for the formation of the [30-51; 14-38] mixed disulfide species. The notation 5/55ssG indicates that the intermediate contains a mixed disulfide with glutathione on Cys^5 or Cys^{55}, or both. The [30-51; 14-38] mixed disulfide species could be generated from the [30-51] intermediate either by (i) oxidation of the 14-38 disulfide bond followed by formation of a mixed disulfide on Cys^5 or Cys^{55} or both (solid lines) or (ii) formation of the mixed disulfide on Cys^5 or Cys^{55} or both in the [30-51] intermediate, followed by oxidation of the 14-38 disulfide (broken lines). The latter route is possible even if the free thiols of Cys^5 and Cys^{55} are completely unreactive in the [30-51; 14-38] intermediate (indicated by **X**).

fast rate of oxidation of the 14-38 disulfide bond in [30-51] does not require that this intermediate have a totally native-like conformation. For example, because Cys^{14} and Cys^{38} are at one end of an antiparallel β-sheet in native BPTI, the rate of forming the 14-38 disulfide bond might be fast if this antiparallel β-sheet were present, even if other parts of the protein were disordered. Indeed, a peptide model of the [30-51] intermediate (*11*), called PαPβ, folds into a native structure that includes this antiparallel β-sheet and the α-helix of BPTI, even though PαPβ lacks half of the residues in BPTI. Direct structural information of an intact model of the [30-51] intermediate in which the remaining Cys residues are mutated to alanine, based on 2D-NMR studies, will be presented elsewhere (*12*).

Finally, previous studies do not provide positive evidence that the thiols of Cys^5 and Cys^{55} in the native intermediate [30-51; 14-38] are normally reactive to disulfide reagents like glutathione. The observation (*3*, *13*) that [30-51; 14-38] accumulates during kinetic folding studies as a mixed disulfide species, in which glutathione is disulfide-linked to Cys^5 or Cys^{55} or both, led to the conclusion that the free thiols in [30-51; 14-38] can react readily with glutathione (*14*). However, it is possible (Fig. 3) for the mixed disulfide to form first in an intermediate with exposed thiols and then to accumulate in a later intermediate, even if the free thiols in the later intermediate are completely inaccessible [contrast with (*14*)]. For example, in the case of [30-51; 14-38], the mixed disulfide species might be generated initially in the one-disulfide intermediate [30-51] (Fig. 3). Oxidation of the 14-38 disulfide bond could then produce the [30-51; 14-38] mixed disulfide species. This explanation is supported by earlier BPTI folding studies (*3*, *13*) showing that mixed disulfide species of the [30-51] intermediate also accumulate in kinetic folding experiments. Indeed, a recent x-ray crystallography study by Kossiakoff (*15*) has shown that a recombinant model of the [30-51; 14-38] intermediate has the same global fold as native BPTI and that the side chains of Val^5 and Ala^{55} (replacing Cys^5 and Cys^{55}, respectively) are buried in the hydrophobic core of the molecule. In addition, working with pure, reversibly trapped [30-51; 14-38], we have shown directly and quantitatively that the free thiols of Cys^5 and Cys^{55} react with glutathione at a greatly reduced rate (16). A similar situation has been reported by Goto and Hamaguchi (*17*) for an immunoglobulin light chain folding intermediate.

In conclusion, our studies (*1*) demonstrate that the previous emphasis on nonnative species as the determining factor for the kinetic properties of BPTI folding was unwarranted. Rather, the essential features of the BPTI folding reaction, including the rearrangement step, are determined in large part by native structure in the observed folding intermediates. Subsequently, it has been demonstrated that the native fold of BPTI can be obtained without nonnative disulfide species. BPTI containing only the 5-55 disulfide bond (with all other Cys residues replaced by Ala or Ser, so that nonnative disulfide bonds cannot form) is folded completely into a native conformation as determined by 2D-NMR (*18*, *19*) and is a functional trypsin inhibitor (*18*). At this point, there is no compelling reason to say that the amino acid sequence of BPTI encodes for nonnative interactions that are important for protein folding. Both kinetic (*1*) and structural (*11*, *15*, *18–21*) studies of folding intermediates support our conclusion that native interactions play a predominant role in the folding of BPTI.

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8. Following the nomenclature in Fig. 2, the rate of II → [30-51; 5-55] was reported to be 5×10^{-3} s^{-1} (*7*). The rate of the intramolecular step of I → [30-51; 5-55] was estimated by Creighton (*3*) also to be 5×10^{-3} s^{-1} and was reported more recently by Goldenberg (*4*) to be 2×10^{-3} s^{-1}.
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22. We thank L. Stryer, B. Tidor, A. Frankel, and A. Cochran for helpful comments on an earlier draft of this response. We are also grateful to A. Kossiakoff for sharing his results before publication.

24 October 1991; accepted 4 December 1991