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.

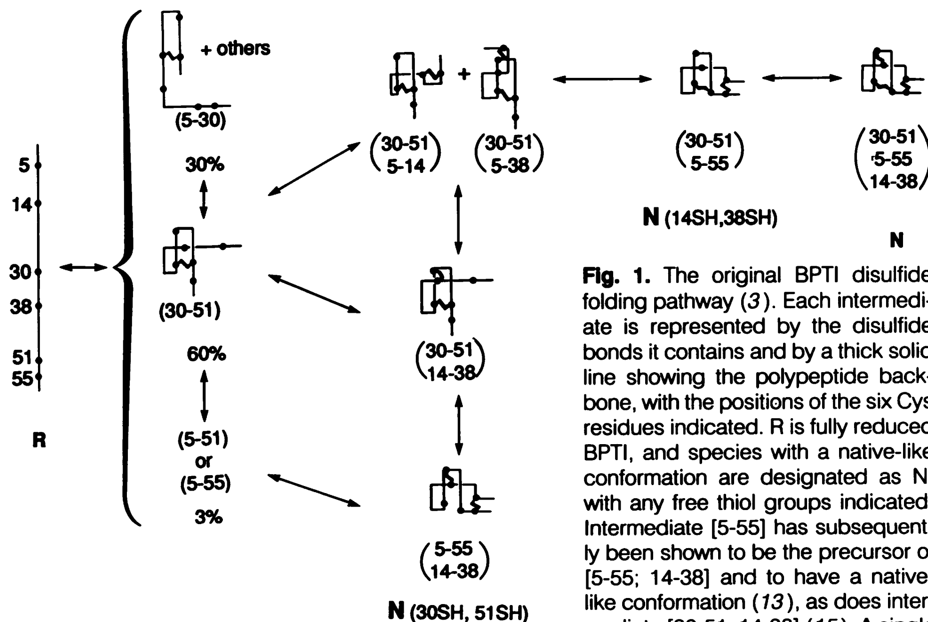

Fig. 1. The original BPTI disulfide folding pathway (*3*). Each intermediate is represented by the disulfide bonds it contains and by a thick solid line showing the polypeptide backbone, with the positions of the six Cys residues indicated. R is fully reduced BPTI, and species with a native-like conformation 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*)]

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

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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] $\not\leftrightarrow$ [30-51; 5-55] and [30-51; 14-38] $\not\leftrightarrow$ 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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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-

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