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Kinetic role of nonnative species in the folding of bovine pancreatic trypsin inhibitor

JONATHAN S. WEISSMAN† AND PETER S. KIM‡

Howard Hughes Medical Institute, Whitehead Institute for Biomedical Research, Departments of †Physics and ‡Biology, Massachusetts Institute of Technology, Nine Cambridge Center, Cambridge, MA 02142

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ABSTRACT **We have shown previously that during the oxidative folding of bovine pancreatic trypsin inhibitor only intermediates with native disulfide bonds are well populated. Nevertheless, these studies also confirmed the earlier conclusion [Creighton, T. E. (1977) *J. Mol. Biol.* 113, 275–293] that the rate-limiting transition in the kinetically preferred route for folding involves intramolecular disulfide bond rearrangements. Consequently, intermediates with nonnative disulfide bonds must form transiently during folding. Two specific nonnative species, denoted [30-51; 5-14] and [30-51; 5-38], in which numbers indicate residues participating in a disulfide bond, can be detected at low levels in kinetic folding experiments with bovine pancreatic trypsin inhibitor. By working with purified reversibly trapped intermediates, the role of these two nonnative species has been examined directly. These species are found to be in relatively rapid exchange with each other and with an initially formed native two-disulfide intermediate [30-51; 14-38]. Thus, the low abundance of the two nonnative species detected in kinetic folding experiments reflects primarily their low thermodynamic stability as compared to this native intermediate. To a small extent, these nonnative species form the productive native intermediate [30-51; 5-55], which is the immediate precursor to the native protein. However, an equal amount of [5-55; 14-38], a nonproductive dead-end intermediate, is also produced. Thus, the nonnative species detected during the folding of bovine pancreatic trypsin inhibitor are not committed to forming the productive native intermediate, nor do they serve to direct folding specifically toward a productive route.**

The native structure of bovine pancreatic trypsin inhibitor (BPTI) is stabilized by three disulfide bonds between residues 30–51, 5–55, and 14–38 (Fig. 1*A*). Upon reduction of these disulfide bonds, BPTI unfolds spontaneously. Thus, protein folding is linked thermodynamically to disulfide bond formation. The folding of BPTI (Fig. 1*B*) has been described in terms of the disulfide-bonded intermediates that accumulate during the oxidative folding of the protein (8, 9). These intermediates are denoted in brackets by the cysteine residues involved in disulfide bonds; native BPTI (N) is thus [30-51; 5-55; 14-38]. Recently, it has been demonstrated that only intermediates with native disulfide bonds are well populated during folding (8).

When BPTI folds in strongly oxidizing conditions, roughly one-half of the molecules form a very stable quasinative intermediate, [5-55; 14-38], that has been designated N* (8, 10, 11). N* has a structure that is essentially identical to that of native BPTI (12, 13). This native structure buries Cys^{30} and Cys^{51} (Fig. 1*A*), rendering them inaccessible to oxidizing agents and, thereby, preventing formation of the final disulfide bond. Because N* neither oxidizes nor rearranges on the time scale of most folding experiments, including those reported here, routes of folding that lead to N* are referred to as "nonproductive."

In the predominant "productive" route for folding, native BPTI is not formed by a simple sequential acquisition of native disulfide bonds (8, 9, 14). Instead, folding proceeds as follows (Figs. 1*B* and 2): (*i*) The native two-disulfide intermediate [30-51; 14-38] accumulates readily to high levels. The [30-51; 14-38] intermediate is designated N′ because nuclear magnetic resonance (NMR) (8, 15) and x-ray crystallographic studies (A. A. Kossiakoff, personal communication) indicate that, despite the absence of the 5-55 disulfide bond, this intermediate folds to a stable structure that is very similar to native BPTI. (*ii*) N′ rearranges to a more stable native two-disulfide intermediate, [30-51; 5-55], known as N^{SH}_{SH}. The rate of rearrangement of N′ to N^{SH}_{SH} is independent of the concentration of oxidizing agents (9), indicating that this transition occurs by intramolecular thiol–disulfide exchange. In addition, the rearrangement of N′ to N^{SH}_{SH} is rate-limiting in the productive folding of BPTI (8, 11). (*iii*) Finally, the 14-38 disulfide bond is oxidized readily in N^{SH}_{SH} to form native BPTI (N).

It has not yet been established firmly why the 5-55 disulfide bond cannot be formed readily in N′ to yield native BPTI (Fig. 2). It seems likely (8), however, that a large factor in the failure of N′ to oxidize directly is that the native structure of this intermediate buries the thiols of Cys^5 and Cys^{55}. Before a new disulfide is formed, a free thiol in a protein first forms a mixed disulfide with an external oxidizing agent, typically the oxidized form of glutathione. By burying thiols, native structure in N′ is expected to inhibit formation of the mixed disulfide species. Other factors, such as the structure of the transition state (18), also may contribute to the failure of N′ to oxidize directly.

We focus here on the mechanism of the rearrangement of N′ to N^{SH}_{SH}. Because only a single cysteine in a disulfide bond can be replaced per thiol–disulfide exchange step, the intramolecular rearrangement of N′ to N^{SH}_{SH} requires at least two steps (Fig. 2). Moreover, the intermediate produced after the first exchange step must contain a disulfide bond not found in native BPTI.

The presence of a nonnative species in the predominant route of productive folding of BPTI is, therefore, required by disulfide chemistry. There is no reason *a priori* that these nonnative species must also play a specific role in directing the folding process. It is possible, however, that the nonnative species contain specific structural information that favors a productive route, disfavors an unproductive route, or otherwise guides the flux of molecules in the folding process.

Two specific nonnative species, [30-51; 5-14] and [30-51; 5-38], are conspicuously present during the folding of BPTI, albeit at low levels compared to the native intermediates (8). These nonnative species are of particular interest (9) because they could serve as direct intermediates in the productive rearrangement of N′ to N^{SH}_{SH} (Fig. 2). For example, [30-51;

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Abbreviation: BPTI, bovine pancreatic trypsin inhibitor.

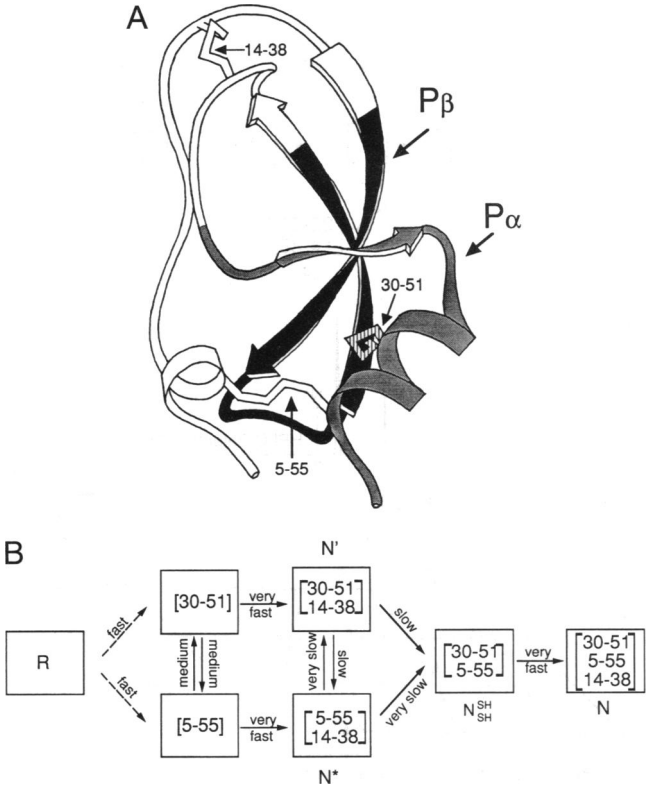

FIG. 1. (*A*) Schematic representation of the crystal structure (1–3) for BPTI (adapted from ref. 4). The three disulfide bonds are denoted. Disulfide bond 14-38 is accessible to solvent, exposing 48% of its total surface area, whereas the disulfide bonds 30-51 and 5-55 are inaccessible, exposing 0% of their total surface area (5, 6). The shaded regions indicate the portions of BPTI corresponding to an autonomously folding subdomain, called PαPβ, that is composed of the peptides Pα and Pβ joined by the 30-51 disulfide bond (7). (*B*) Schematic representation of the kinetically preferred pathway for the folding of BPTI (8). R denotes the reduced protein. All of the well-populated intermediates contain only native disulfide bonds. Qualitative descriptions of the relative rates of the intramolecular transitions associated with each step at pH 7.3 and 25°C are indicated. These rates were determined directly (8) starting with pure reversibly trapped intermediates. N* is a dead-end nonproductive intermediate that is stable for weeks under these conditions (see text). 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].

14-38] (i.e., N′) could rearrange to [30-51; 5-14] in a single thiol–disulfide exchange step, and this nonnative species could then proceed to [30-51; 5-55] (i.e., N^{SH}_{SH}) in a second step. The importance of [30-51; 5-14] and [30-51; 5-38] is suggested also by the observation (14, 19) that when both Cys^{14} and Cys^{38} are blocked chemically or replaced by mutagenesis, thereby preventing the formation of both [30-51; 5-14] and [30-51; 5-38], N^{SH}_{SH} does not form readily in the presence of weak oxidizing agents like the oxidized form of dithiothreitol.§ Largely on the basis of these considerations, it has been proposed (14) that [30-51; 5-14] and [30-51; 5-38] play a critical role in the formation of the productive intermediate N^{SH}_{SH}.

§Nonetheless, the rate of the intramolecular step for formation of [30-51; 5-55] in the Cys^{14}/Cys^{38} mutant is almost identical to that of the wild-type protein (14, 19). Consequently, the rates of folding to [30-51; 5-55] in the presence of strong oxidizing agents like oxidized glutathione in the wild-type protein and the Cys^{14}/Cys^{38} mutant are similar (20), suggesting that the nonnative species do not accelerate folding (for a more complete discussion, see ref. 21).

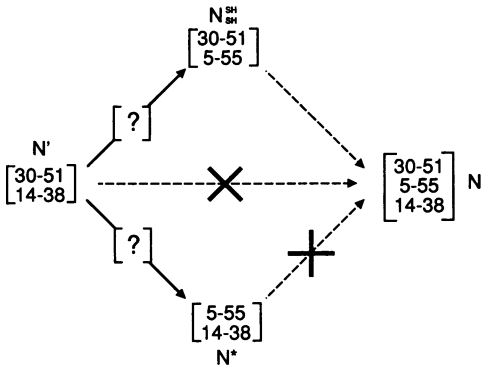

FIG. 2. Diagram of the final steps in the folding of BPTI. Steps involving formation of additional disulfide bonds are indicated by dashed lines. Steps involving intramolecular rearrangement of existing disulfide bonds are indicated by solid lines. [?] denotes that the indicated rearrangement step must proceed through at least one nonnative species. × denotes that the indicated step does not occur to a significant extent. Each of the native two-disulfide intermediates (N′, N^{SH}_{SH}, and N*) is known to be folded completely into an essentially native structure (refs. 12, 13, and 15–17; A. A. Kossiakoff, personal communication), but only N^{SH}_{SH} can be oxidized readily to native BPTI (N). The rate-limiting transition in the productive folding of BPTI is the intramolecular rearrangement of N′ to N^{SH}_{SH}.

On the other hand (8, 11), N′ rearranges to the nonproductive intermediate N* as rapidly as it rearranges to the productive intermediate N^{SH}_{SH}. Since the formation of N* (Fig. 2) requires at least one intermediate that is distinct from both [30-51; 5-14] and [30-51; 5-38], the similarity in these two rates suggests that the rearrangement process is largely random (8). The simplest explanation for this randomness is that both rearrangements require substantial unfolding of N′. This explanation is supported by the observation that, even at high concentrations (6 M), the chemical denaturant urea accelerates the rearrangement of N′ (8). These considerations suggest that the nonnative species [30-51; 5-14] and [30-51; 5-38] do not direct folding specifically toward a productive route.

Because the thiolate anion is the reactive species in thiol–disulfide exchange, it is possible to quench folding rapidly by lowering the pH. Protonation is reversible, however, so acid-quenched intermediates must be separated rapidly and at low pH to avoid further oxidation or rearrangement. The validity of using acid-quenching and reversed-phase HPLC at pH 2 for studies of BPTI folding has been established (8). A substantial advantage of acid-quenching is that it is reversible. As a result, it is possible to purify an acid-quenched intermediate to homogeneity and subsequently to allow further rearrangement or folding to occur (8). By taking advantage of the reversibility of acid-quenching, we have determined directly the kinetic roles of the nonnative species [30-51; 5-14] and [30-51; 5-38] in the folding of BPTI.

MATERIALS AND METHODS

Purification of Acid-Quenched Intermediates. Acid-quenched [30-51; 5-14] and [30-51; 5-38] were produced as follows: (*i*) Reduced BPTI (final concentration, 30 μM) was oxidized for 5 min with the oxidized form of glutathione (150 μM) at pH 8.7 (see ref. 8 for oxidation details); the oxidation was quenched with 0.1 vol of 3 M HCl. (*ii*) Components of the oxidation mixture were purified on a Vydac C_{18} preparative column (stock 218TP1022) heated to 50°C. A linear acetonitrile/H_2O gradient in the presence of 0.1% trifluoroacetic acid was used. (*iii*) The peaks containing [30-51; 5-38] and [30-51; 5-14] were purified further on a Vydac C_{18} semi-preparative column (stock 218TP510) heated to 37°C. The purified intermediates were then stored in a lyophilized form

in a desiccator until a rearrangement reaction was initiated. The identity of the disulfide species was determined as described in ref. 8.

Rearrangement of Purified Intermediates. Rearrangement reactions were initiated by the addition of folding buffer (100 mM sodium phosphate, pH 7.0/150 mM NaCl/1 mM EDTA) to lyophilized intermediates in an anaerobic chamber (Coy Laboratory Products, Ann Arbor, MI). The rearrangement reactions were carried out in a circulating water bath at 25°C. Folding buffer was degassed by stirring in the anaerobic chamber for several days prior to the experiment (<25% of a 3 μM N^{SH}_{SH} solution oxidizes to native BPTI in 45 h under these conditions). The concentration of intermediates in the rearrangement mixture was $\approx$2 μM. The relative ratios of N', N*, and N^{SH}_{SH} produced during the rearrangement of [30-51; 5-38] and [30-51; 5-14] were the same when the concentration of intermediates in the rearrangement mixture was increased to 10 μM. Aliquots were removed from the rearrangement mixture at the appropriate time, quenched with 0.1 vol of 3 M HCl, and analyzed by HPLC (8) with a Vydac C_{18} analytical column (stock 218TP54) heated to 37°C. Buffer A is 0.1% trifluoroacetic acid in water, and buffer B is 0.1% trifluoroacetic acid/90% (vol/vol) acetonitrile/10% (vol/vol) water. In the experiments depicted in Fig. 3, a gradient with linear segments was used: 0 min, 90% buffer A; 15 min, 73% buffer A; 35 min, 71% buffer A; 50 min, 70% buffer A; 70 min, 69% buffer A; 90 min, 66% buffer A.

Determination of Rate Constants. The rates of interconversion among [30-51; 5-14], [30-51; 5-38], and [30-51; 14-38] were determined by analyzing the initial time points in the rearrangements of [30-51; 5-14] and [30-51; 5-38]. When starting with intermediate A, the rate of transition from A to B ($k_{A\rightarrow B}$) is given by:

$$k_{A\rightarrow B} = \frac{\frac{\partial[B]}{\partial t}(t=0)}{[A]}.$$

In these studies, $\partial[B]/\partial t$ ($t = 0$) was estimated by monitoring the concentration of B at several time points between 1 and 10 sec. Simulations with computer-generated data indicate that this introduces an error of <20%. The initial rate measurements could be distorted significantly if there were a rapidly rearranging impurity in the purified acid-quenched species. To check for this possibility, purified [30-51; 5-14] or [30-51; 5-38] was allowed to rearrange for 30 sec at pH 7.0. During this time, $\approx$40% of the starting material rearranged. The remaining [30-51; 5-14] or [30-51; 5-38] was then purified again from the rearrangement mixture by HPLC. Rapidly rearranging contaminants are expected to be depleted selectively by this procedure. The initial rates measured with the twice-purified material were not significantly different from the rates obtained with the once-purified material.

RESULTS

The time course of rearrangement of the two nonnative species [30-51; 5-14] and [30-51; 5-38] was determined by initiating a folding reaction with the addition of buffer at pH 7.0 to the chromatographically purified reversibly trapped intermediates, waiting for a defined period of time, and quenching the reaction with acid. The spectrum of intermediates present at each time point was determined by HPLC. Because the rate-limiting transition in the folding of BPTI involves only intramolecular thiol–disulfide exchange and, therefore, is not influenced by external disulfide reagents (9), the folding reactions were carried out in the absence of disulfide reagents.

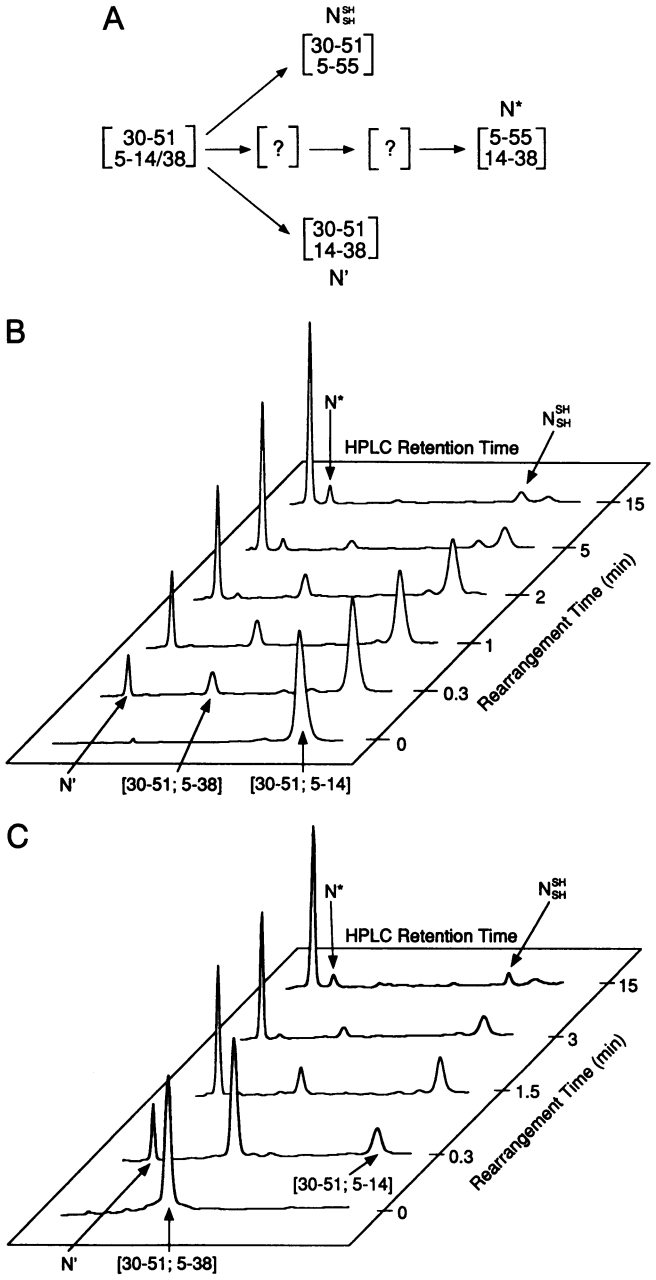

FIG. 3. Rearrangements of the nonnative species. (*A*) Diagram of the steps in the rearrangement of the nonnative species. [30-51; 5-14/38] represents both [30-51; 5-14] and [30-51; 5-38]. Only rearrangements with a native intermediate as the final product are depicted; these native intermediates are all stable on the time scale of the experiments. The number of arrows indicates the minimal number of thiol–disulfide exchange steps required for the indicated transition. [?] denotes that at least one nonnative species is required at the indicated step. In the rearrangement to N*, one of the nonnative species must be a two-disulfide molecule in which both disulfides are nonnative (see text). (*B*) Rearrangement of [30-51; 5-14]. Acid-quenched HPLC-purified [30-51; 5-14] was allowed to rearrange by raising the pH to 7.0 in the absence of redox reagents (8). At the indicated times, a portion of the sample was removed, quenched with acid and analyzed by HPLC. (*C*) Rearrangement of [30-51; 5-38].

In each case, the rearrangement process was monitored for 15 min (Fig. 3). It is important to note that on this time scale, secondary rearrangements of the three native intermediates (N', N*, and N^{SH}_{SH}) are not significant. N' rearranges (to N* and N^{SH}_{SH}) at a rate of 1.3×10^{-3} min^{-1} at 25°C and pH 7.0 (data not shown). Thus, <2% of N' will rearrange in 15 min. The

other two native intermediates (N* and N^{SH}_{SH}) do not rearrange to a detectable extent in 15 min.

The nonnative species [30-51; 5-14] rearranged preferentially to both [30-51; 5-38] and N′ on the minute time scale (Fig. 3*B*). During this rearrangement, a small amount (≈10%) of the starting material formed N^{SH}_{SH}. However, an equal amount of the nonproductive intermediate N* was also produced (Fig. 3*B*). No other intermediates accumulated significantly during the rearrangement of [30-51; 5-14]. The rearrangement of [30-51; 5-38] (Fig. 3*C*), although ≈3-fold faster than that of [30-51; 5-14], was qualitatively very similar. The [30-51; 5-38] species rearranged preferentially to both [30-51; 5-14] and N′. Approximately 10% of the starting material formed N^{SH}_{SH}. Again, however, an equal amount of N* was also produced (Fig. 3*C*).

These experiments demonstrate that the nonnative species [30-51; 5-14] and [30-51; 5-38] are not committed to forming the productive intermediate N^{SH}_{SH}. Although [30-51; 5-14] and [30-51; 5-38] can form either N^{SH}_{SH} or N′ with a single thiol–disulfide exchange step (Fig. 3*A*), rearrangement to N′ occurs ≈10 times more rapidly. Even more striking is that the transition of these nonnative species to either N* or N^{SH}_{SH} occurs at the same rate.

Since N′ is stable on the time scale of these experiments, the transition of these nonnative species to N* does not proceed through N′. As a consequence, the rearrangement to N* from [30-51; 5-14] or [30-51; 5-38] must proceed with a minimum of three thiol–disulfide exchange steps (Fig. 3*A*). Moreover, at least one of the intermediates in this rearrangement must be a two-disulfide species in which both disulfide bonds are nonnative. It is extremely unlikely, therefore, that any significant structure persists throughout the transition to N*. The similarity in the rate of formation of N^{SH}_{SH} and N* suggests strongly that formation of the productive intermediate N^{SH}_{SH} also requires essentially complete unfolding of structure (cf. refs. 18, 22, and 23).

The preferential interconversions among the two nonnative species [30-51; 5-14] and [30-51; 5-38] and the native intermediate N′ (Fig. 3) made it possible for us to measure directly the individual rate constants for the interconversions among these three species (k_1, k_2, k_3, and k_4 in Fig. 4*A*). It is possible that other undetected, but kinetically important, nonnative species are present during the rearrangement of [30-51; 5-14] and [30-51; 5-38]. These species do not accumulate substantially, however, so the transitions among [30-51; 5-38], [30-51; 5-14], and N′ can still be described by single rate constants. These measured rate constants were then used to predict the entire time course of rearrangement for each of the two nonnative species. For both nonnative species, the predicted time course agrees well with the measured data (Fig. 4 *B* and *C*). Since the values of k_1, k_2, k_3, and k_4 were determined directly from the initial rate measurements (i.e., in contrast to fitting the rate constants to the overall kinetics of rearrangement), the quality of the agreement between the predicted and measured time courses supports strongly the accuracy of these measured rate constants and the validity of the model depicted in Fig. 4*A*.

DISCUSSION

Because the nonnative species [30-51; 5-14] and [30-51; 5-38] and the native intermediate N′ are in rapid equilibrium, the relative abundance of these species in kinetic folding experiments reflects their relative thermodynamic stabilities. At neutral pH, the ratios of N′, [30-51; 5-14], and [30-51; 5-38] in kinetic folding experiments are ≈100:3:1, respectively (ref. 8 and unpublished data). We conclude, therefore, that the nonnative species [30-51; 5-14] and [30-51; 5-38] are ≈30- and 100-fold, respectively, less stable than the native intermediate N′.

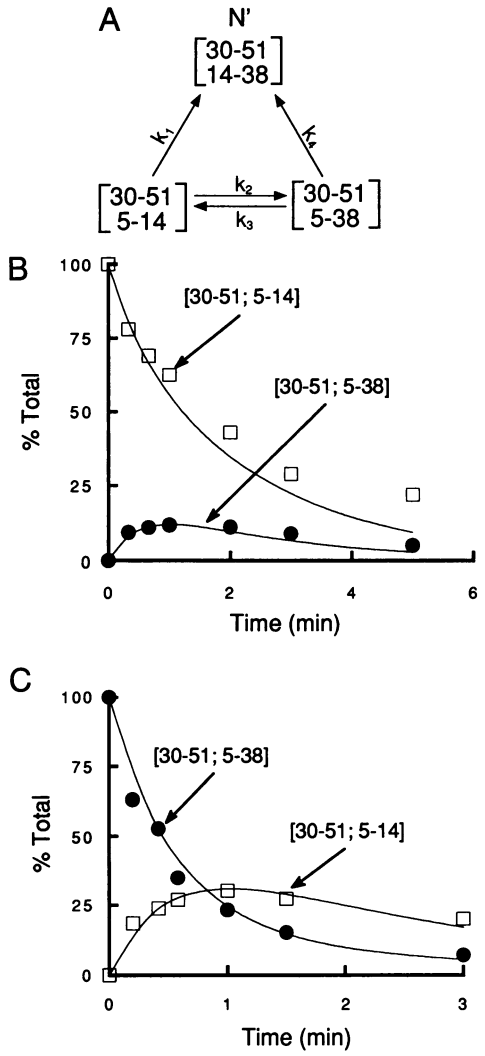

FIG. 4. Quantitative analysis of the rapid interconversions among the two nonnative species and N′. (*A*) Simplified model for the rearrangement of these three species. Rearrangements of N′ are not included in the model because these rearrangements are ≈30-fold slower than the rearrangements of [30-51; 5-14] or [30-51; 5-38]. A more significant limitation of this model is that rearrangements to the native intermediates N* and N^{SH}_{SH} are not included. Approximately 20% of [30-51; 5-14] or [30-51; 5-38] rearranges to these native intermediates. (*B*) Rearrangement of [30-51; 5-14]. The concentrations of [30-51; 5-14] (□) and [30-51; 5-38] (●) present during the rearrangement of [30-51; 5-14] are compared to the predicted values (lines). Predicted curves were generated, by the MATHEMATICA software package (24), for the values of k_1, k_2, k_3, and k_4 determined from the initial rate measurements. These values are as follows: k_1 = 0.35 min^{-1}; k_2 = 0.35 min^{-1}; k_3 = 0.9 min^{-1}; k_4 = 0.7 min^{-1}. (*C*) Rearrangement of [30-51; 5-38]; symbols are as in *B*.

The preferential interconversions among [30-51; 5-14], [30-51; 5-38], and N′ can be rationalized in structural terms if these species share a common native-like subdomain structure (Fig. 1*A*). All three species contain the native 30-51 disulfide bond. A peptide model of the [30-51] intermediate, called PαPβ, forms an autonomously folding subdomain of native structure containing both the α-helix and β-sheet of BPTI (7). The structure of N′ is very similar to that of native BPTI (refs. 8 and 15; A. A. Kossiakoff, personal communication) and includes the PαPβ subdomain. The peptide model PαPβ folds (7) even though the regions of BPTI containing Cys^5, Cys^{14}, and Cys^{38} have been removed (Fig. 1*A*). Thus, it is possible that [30-51; 5-14] and [30-51; 5-38] retain the PαPβ subdomain structure despite the presence of a nonna-

tive disulfide bond. Indeed, recent structural studies of recombinant models of [30-51; 5-14] and [30-51; 5-38] suggest that these species and the native intermediate [30-51] exhibit comparable degrees of stable structure (25). If $P\alpha P\beta$ structure is present in these two nonnative species, Cys^5 would be found at the top of the β-sheet, thereby placing Cys^5, Cys^{14}, and Cys^{38} in proximity to one another. Consequently, $P\alpha P\beta$ structure might be able to persist throughout the transitions of [30-51; 5-14] and [30-51; 5-38] to each other or to N'.

In contrast, our studies suggest strongly that productive rearrangement of the nonnative species [30-51; 5-14] and [30-51; 5-38] to N_{SH}^{SH} is a random process that requires substantial loss of structure. These nonnative species form N* at the same rate as they form N_{SH}^{SH} despite the fact that the transition to N* must proceed through several unobserved nonnative species (Fig. 3*A*). Moreover, it is possible that much of the flux of the rearrangement of N' to either N_{SH}^{SH} or N* proceeds through a route not involving [30-51; 5-14] or [30-51; 5-38].

Nonetheless, our studies do not rule out the possibility that folding proceeds predominantly by direct rearrangement of N' $\rightarrow$ [30-51; 5-14]/[30-51; 5-38] $\rightarrow$ N_{SH}^{SH}. Because the three species N', [30-51; 5-14], and [30-51; 5-38] are in rapid equilibrium, the rate-limiting step in such a direct pathway would be the rearrangement of the two nonnative species to N_{SH}^{SH}. The observation, however, of a rapid equilibrium between N' and the two nonnative species does rule out a route for folding that circumvents N', such as [30-51] $\rightleftharpoons$ [30-51; 5-14] $\rightleftharpoons$ N_{SH}^{SH}, or [30-51] $\rightleftharpoons$ [30-51; 5-38] $\rightleftharpoons$ N_{SH}^{SH}.

In conclusion, we find no evidence that nonnative structures play a significant role in directing the folding of BPTI, even though the predominant route for forming native BPTI requires the transient formation of species with nonnative disulfide bonds (14). Earlier investigations have (*i*) demonstrated that all well-populated intermediates in the folding of BPTI contain only native disulfide bonds (8), (*ii*) identified stable subdomains of native BPTI structure (4, 7), and (*iii*) shown that N' folds to a stable structure that is almost identical to that of native BPTI (refs. 8 and 15 and A. A. Kossiakoff, personal communication) even though this native intermediate is formed prior to the rearrangement step requiring nonnative species. The presence of kinetically important nonnative species in the oxidative folding of BPTI is, therefore, predominantly a consequence of the requirements of disulfide chemistry.

Other studies demonstrate unequivocally that the native fold of BPTI can be obtained without the assistance of nonnative disulfide species. BPTI containing only the 5-55 disulfide bond (with all other cysteine residues replaced by alanine or serine, so that nonnative disulfide bonds cannot form) is folded completely into a native conformation as determined by two-dimensional NMR (26, 27) and is a functional trypsin inhibitor (27). Nonnative interactions might yet prove to be important in protein folding reactions; for example, they may stabilize early "molten globule"-like states (28, 29). However, studies of BPTI argue strongly that native interactions play a predominant role in determining protein folding.

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