Proc. Natl. Acad. Sci. USA
Vol. 89, pp. 1519–1523, March 1992
Biochemistry

Complete folding of bovine pancreatic trypsin inhibitor with only a single disulfide bond

(protein folding)

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Communicated by Frederic M. Richards, November 4, 1991 (received for review September 3, 1991)

ABSTRACT **In the oxidative folding of bovine pancreatic trypsin inhibitor (BPTI) at neutral pH, only two one-disulfide intermediates accumulate to a significant extent, namely [5-55] and [30-51]. In this paper we describe a recombinant model of [5-55], designated $[5\text{-}55]_{Ala}$, which was made by replacing the cysteine residues not involved in the disulfide bond with alanine. As judged by two-dimensional NMR, $[5\text{-}55]_{Ala}$ folds into essentially the same conformation as native BPTI. Moreover, like native BPTI, $[5\text{-}55]_{Ala}$ inhibits trypsin stoichiometrically. Thus, the disulfide-bonded intermediate [5-55] corresponds not to a partially folded protein folding intermediate but rather to an essentially completely folded protein. This conclusion provides an explanation for many of the thermodynamic and kinetic properties of [5-55] in the folding pathway of BPTI.**

Bovine pancreatic trypsin inhibitor (BPTI) unfolds upon reduction of its three disulfide bonds (Fig. 1*A*). This process is reversible, as oxidation of reduced BPTI results in formation of native protein. Thus, for BPTI, protein folding is coupled to disulfide bond formation (7, 13). During the oxidative folding of BPTI at neutral pH, only five disulfide-bonded intermediates accumulate to a substantial extent (Fig. 1*B*), and each contain only native disulfide bonds (7).

Thermodynamic linkage requires that disulfide bond formation is coupled to protein stability. There is no requirement *a priori*, however, that disulfide-bonded intermediates correspond to partially folded states. For example, RNase T1 maintains a native structure, as determined by enzymatic activity and fluorescence, even after reduction of both disulfide bonds (14), which together stabilize the protein by more than 8 kcal/mol (1 kcal = 4.18 kJ). Nevertheless, as protein folding intermediates have been difficult to isolate and characterize (15, 16), one would like to know if the disulfide-bonded intermediates that accumulate in the oxidative folding of BPTI correspond to partially folded states.

The three major two-disulfide intermediates that accumulate at neutral pH (Fig. 1*B*) do not correspond to partially folded states. They have all been shown to be folded essentially into the same conformation (refs. 9–12; A. Kossiakoff, personal communication) as native BPTI, though they are less stable. It has remained to be determined whether the major one-disulfide intermediates, [5-55] and [30-51] (Fig. 1*B*), correspond to partially folded states.

A synthetic peptide model (17) of [5-55], named PαPγ, has been made that contains two-thirds of the residues in BPTI; cysteines not involved in the disulfide bond have been replaced with alanines. As judged by NMR, PαPγ folds into a close-packed native structure (17) with a melting temperature of ≈28°C. A recombinant model of [5-55] has also been made (18) in which the cysteines not involved in the disulfide bond have been replaced with serines. This recombinant model folds with a melting temperature (18) of ≈15°C, but it is unfolded at 25°C, the temperature at which [5-55] is observed during folding (7). The instability of this model may

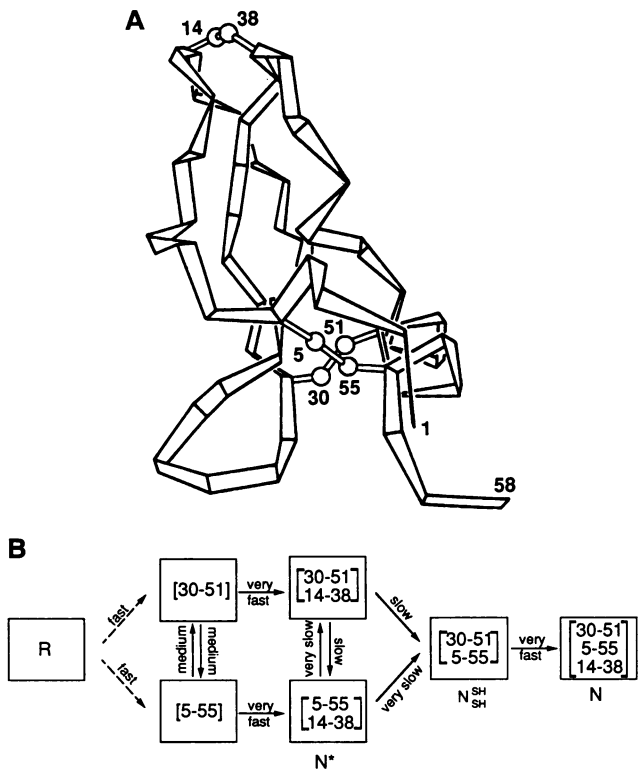

FIG. 1. (*A*) Schematic representation of the crystal structure (1–3) for BPTI (adapted from ref. 4). The first and last residues and the cysteine residues involved in disulfide bonds are labeled. The disulfide bond 14-38 is accessible to solvent, exposing 48% of its total surface area, whereas the disulfide bonds 30-51 and 5-55 are inaccessible, exposing 0% of their total surface area (2, 5, 6). (*B*) Schematic diagram of the folding pathway of BPTI at 25°C, pH 7.3 (from ref. 7). Intermediates are designated by the disulfide bonds that they contain. R refers to reduced BPTI; N, to native BPTI; N^{SH}_{SH}, to the precursor to native BPTI; and N*, to a kinetically trapped intermediate. The dashed arrows indicate that the major one-disulfide intermediates do not form directly from reduced protein but rather from rearrangement of other one-disulfide intermediates (8). Estimates for the relative rates of intramolecular transitions at pH 7.3 are indicated (7); "very fast" rates are on the order of milliseconds, while "very slow" rates are on the order of months. N^{SH}_{SH}, N*, and [30-51; 14-38] fold into essentially the same conformation (refs. 9–12; A. Kossiakoff, personal communication) as native BPTI.

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Abbreviations: BPTI, bovine pancreatic trypsin inhibitor; COSY, two-dimensional correlated spectroscopy; NOE, nuclear Overhauser effect; NOESY, two-dimensional nuclear Overhauser spectroscopy; TOCSY, two-dimensional total correlation spectroscopy.

arise from the Cys → Ser substitutions and/or the Met-52 → Arg substitution that was also introduced (18).

We have made a different recombinant model of [5-55] in which the remaining cysteines have been replaced with alanines. Expression of this model in *Escherichia coli* results in the addition of a methionine residue at the amino terminus. This recombinant model, designated $[5\text{-}55]_{Ala}$, folds with substantial stability, having a melting temperature of ≈40°C. As judged by two-dimensional NMR, $[5\text{-}55]_{Ala}$ folds into essentially the same conformation as native BPTI. Moreover, $[5\text{-}55]_{Ala}$ inhibits trypsin stoichiometrically. These results indicate that the disulfide-bonded intermediate [5-55] is not a folding intermediate in a structural sense.

MATERIALS AND METHODS

Plasmid Construction. To produce Cys → Ala mutants of BPTI, we first constructed a plasmid that utilizes a T7 expression system (19) to produce wild-type BPTI, named M-BPTI because *E. coli* adds a Met residue to the amino terminus. The gene for M-BPTI was synthesized with convenient restriction sites and optimal codons (20) for *E. coli*. The gene was ligated into the *Nde* I/*Bam*HI site (19) of pET-3a. A plasmid that codes for the mutant $[5\text{-}55]_{Ala}$ was constructed by mutating Cys-14, Cys-30, Cys-38, and Cys-51 to Ala by cassette mutagenesis.

Protein Expression. To express (19) $[5\text{-}55]_{Ala}$, we used the *E. coli* strain BL21(DE3)-pLys(S). Freshly transformed cells were grown at 37°C in Luria broth and were induced, after reaching an OD_{590} of 1.0, by adding isopropyl β-D-thiogalactopyranoside to 0.4 mM. After 20 min, rifampicin was added to 150 μg/ml; this increased expression of $[5\text{-}55]_{Ala}$ but not M-BPTI. After another 40 min, the cultures were spun at 2000 × *g* for 30 min. The resulting cell pellets were frozen.

Purification. M-BPTI forms inclusion bodies during expression, so even unstable mutants can be expressed. Inclusion bodies of $[5\text{-}55]_{Ala}$ were purified as follows. Cell pellets were sonicated on ice in 50 mM Tris·HCl, pH 8.7/15% (vol/vol) glycerol/100 nM $MgCl_2$/10 nM $MnCl_2$ containing DNase I at 10 μg/ml and then spun at 20,000 × *g* for 20 min. The resulting pellet was sonicated in 50 mM Tris·HCl, pH 8.7/1 mM EDTA/1% Nonidet P-40/1% deoxycholic acid and spun at 20,000 × *g* for 20 min. The resulting pellet was dissolved in 0.2 M Tris·HCl, pH 8.7/6 M guanidine hydrochloride (Gdn·HCl)/0.1 M dithiothreitol and dialyzed against 5% (vol/vol) HOAc. The dialysate was purified by reversed-phase HPLC. Reduced $[5\text{-}55]_{Ala}$ at 150 μM was oxidized by air in 6 M Gdn·HCl/0.2 M Tris·HCl, pH 8.7. Oxidized and reduced $[5\text{-}55]_{Ala}$ were repurified by reversed-phase HPLC. Protein sequencing indicated that a Met was added to the amino terminus of $[5\text{-}55]_{Ala}$, as with M-BPTI. The molecular mass of oxidized $[5\text{-}55]_{Ala}$ was confirmed by fast-atom bombardment mass spectrometry (observed, 6519 Da; calculated, 6519 Da) (M-Scan, West Chester, PA).

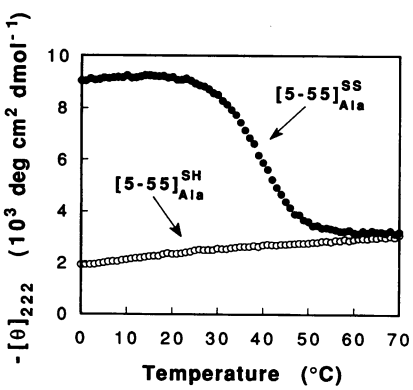

FIG. 2. The recombinant model $[5\text{-}55]_{Ala}$ folds and requires the 5-55 disulfide bond for folding. The temperature dependence of molar ellipticity $[\theta]_{222}$ is shown for $[5\text{-}55]_{Ala}$ (●) and reduced $[5\text{-}55]_{Ala}$ (○). The melting temperature of $[5\text{-}55]_{Ala}$ is ≈40°C, as estimated by the maximum of the first derivative of the temperature dependence of $[\theta]_{222}$.

Table 1. Inhibition of trypsin

Inhibitor	% inhibition at 1:1 molar ratio
Native BPTI	107
$[5\text{-}55]^{SS}_{Ala}$	99
$[5\text{-}55]^{SH}_{Ala}$	4

Error is roughly ± 10%.

Circular Dichroism. Measurements were made in a 10-mm pathlength cell in an Aviv model 62DS circular dichroism spectrometer equipped with a temperature-control unit. Samples, degassed by reduced pressure, contained protein at a concentration of ≈10 μM, as determined by tyrosine and cystine absorbance (21) of stock solutions. Sample buffer was 10 mM sodium phosphate, pH 7.0/150 mM NaCl/1 mM EDTA. The reduced sample also contained 0.2 mM dithiothreitol and was maintained under argon.

Trypsin Inhibition. Inhibition of trypsin activity at 15°C was assayed (22) by monitoring the hydrolysis of N^{α}-benzoyl-DL-arginine *p*-nitroanilide in 0.2 M sodium phosphate, pH 7.3/0.1 M KCl/1 mM EDTA at a trypsin concentration of 250 nM. Inhibitor and trypsin were incubated in buffer 5 min prior to the addition of substrate. Inhibition at stoichiometric concentrations was determined by linear extrapolation of measurements made at six substoichiometric concentrations of inhibitor. The concentrations of $[5\text{-}55]_{Ala}$ and trypsin stock solutions were determined by using tyrosine and cystine absorbance (21); the concentrations of native BPTI stock solutions, using an extinction coefficient (23) of 5400 $cm^{-1} \cdot M^{-1}$ at 280 nm.

NMR. Strong similarity between spectra of $[5\text{-}55]_{Ala}$ and native BPTI facilitated the transfer of assignments (24–26) from native BPTI to $[5\text{-}55]_{Ala}$, which were checked by identifying unambiguous, sequential, nuclear Overhauser effect (NOE) connectivities (27). Spectra of BPTI at 20°C were assigned by transferring assignments (25) from 36°C. Spectra were collected at a protein concentration of ≈5 mM (pH 4.6) in the absence of buffer and salt. Trimethylsilylpropionic acid was used as a standard (28). Data were collected on a Bruker AMX 500-MHz spectrometer, presaturating water for 1.5 s. After four dummy scans, 1024 data points were collected in the t_2 dimension averaging over at least 32 free-induction decays; 256 increments were used in the t_1 dimension. Data from two-dimensional nuclear Overhauser spectroscopy (NOESY) experiments were collected with a mixing time of 150 ms; data from two-dimensional total correlation spectroscopy (TOCSY) experiments, with mixing times of 45 or 110 ms. Two-dimensional correlated spectroscopy (COSY) and TOCSY data were processed by using a Gaussian/Lorentzian window in t_2 and a shifted sine-bell and Kaiser window in t_1; NOESY data, using a shifted sine-bell in t_2 and t_1. In all data sets, the first t_1 was multiplied (29) by 0.5.

RESULTS

The circular dichroism signal of $[5\text{-}55]_{Ala}$ at 222 nm exhibits a sigmoidal dependence on temperature, indicating that $[5\text{-}55]_{Ala}$ folds (Fig. 2). Upon reduction of the disulfide bond in $[5\text{-}55]_{Ala}$, the protein unfolds, indicating that folding is coupled to disulfide bond formation. The melting temperature of $[5\text{-}55]_{Ala}$ is ≈40°C, roughly 10°C higher than the melting temperature of PαPγ, a peptide model (17) of [5-55]. PαPγ contains only two-thirds of the residues in [5-55]; the difference in stability between these two molecules suggests that the additional residues in $[5\text{-}55]_{Ala}$ are folded.

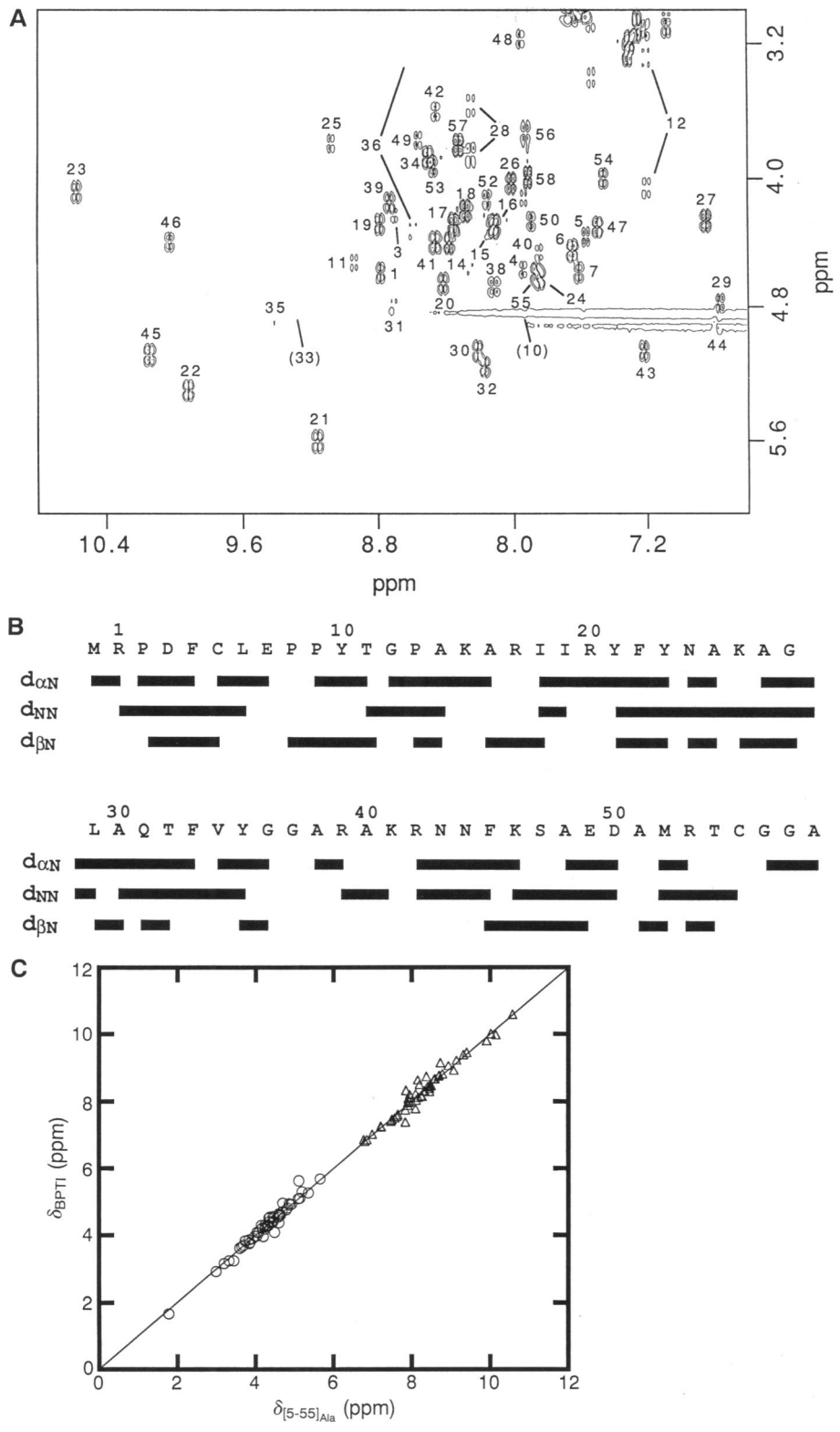

FIG. 3. The chemical shifts of the protons in $[5\text{-}55]_{Ala}$ are very similar to the chemical shifts of the corresponding protons (24–26) in native BPTI, suggesting similar global folds in each. (*A*) The fingerprint region of a COSY spectrum of $[5\text{-}55]_{Ala}$ in H_2O at 20°C, pH 4.6. The assignments of the α-amide cross-peaks are shown. The crosspeaks for Tyr-10 and Phe-33, both near the water resonance, are not observed readily in this spectrum. The cross-peaks for Ala-51 and Gly-37 lie outside of the region plotted. (*B*) Summary of unambiguous sequential NOE connectivities. The sequence of BPTI is shown in the one-letter amino acid code; the methionine added by *E. coli* to the amino terminus is designated residue 0. Bars indicate unambiguous NOEs between the $C^{\alpha}H$, NH, or $C^{\beta}H$ of residue *i* to the NH of residue *i* + 1 as denoted by $d_{\alpha N}$, d_{NN}, and $d_{\beta N}$, respectively. All NOE connectivities are unambiguous in the sense that each NOE could be assigned to only a single pair of protons. (*C*) Correlation is strong between the chemical shifts of backbone protons in $[5\text{-}55]_{Ala}$ and the chemical shifts of the corresponding protons in native BPTI at 20°C. The chemical shifts of all α (○) and all backbone amide (△) protons are shown, with the exception of Phe-33 α and Gly-37 amide protons, which were not assigned for BPTI at 20°C. A line with a slope of 1 is shown.

At 15°C, where $[5\text{-}55]_{Ala}$ is folded (Fig. 2), $[5\text{-}55]_{Ala}$ inhibits trypsin in a stoichiometric manner (Table 1). Under similar conditions, however, reduced $[5\text{-}55]_{Ala}$ does not inhibit trypsin to a significant extent. The binding of $[5\text{-}55]_{Ala}$ to trypsin is strong; given the concentrations used in the assay, the dissociation constant is less than 30 nM.

Similarity between NMR spectra of $[5\text{-}55]_{Ala}$ and native BPTI facilitated the assignment of proton spectra of $[5\text{-}55]_{Ala}$ (Fig. 3*A*). These assignments were checked for internal consistency by identifying unambiguous sequential, NOE connectivities (Fig. 3*B*). All residues exhibited at least one unambiguous sequential NOE, except Gly-37, which was assigned by default. Only one set of cross-peaks is observed in the COSY spectrum (Fig. 3*A*), indicating that $[5\text{-}55]_{Ala}$ exists predominantly in a single conformation at 20°C.

A comparison of the chemical shifts of the backbone α and amide protons in $[5\text{-}55]_{Ala}$ and BPTI indicates a strong correlation between the chemical shifts of these protons (Fig. 3*C*). Protons with the greatest deviation of chemical shift cluster near the alanine substitutions or near the added

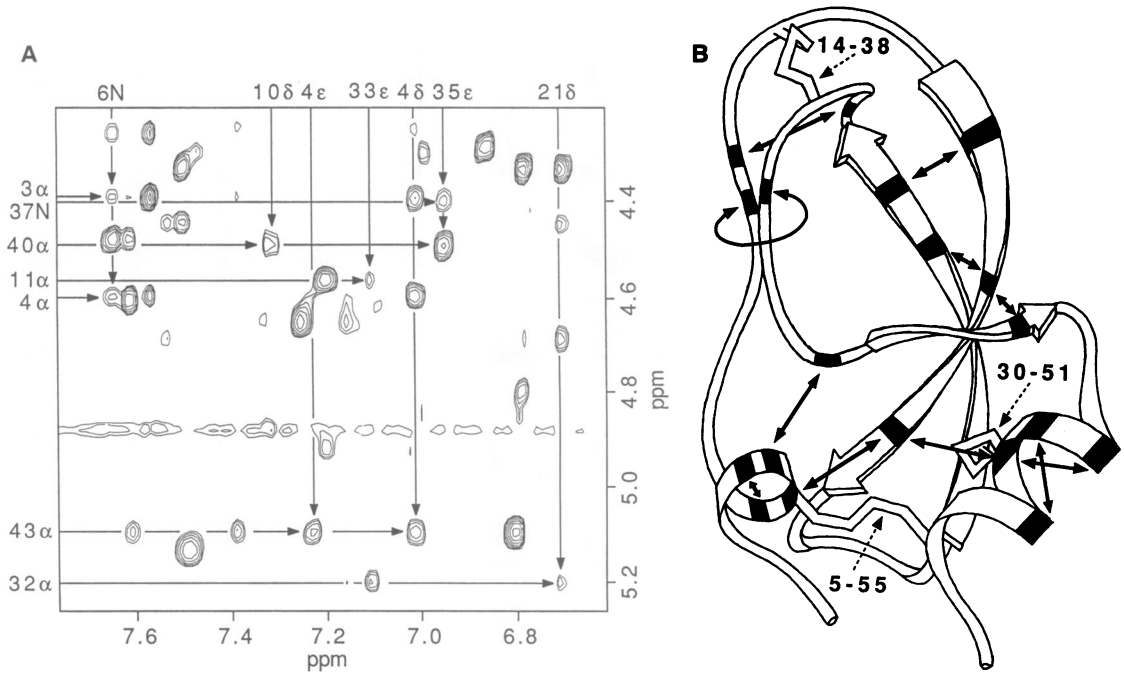

FIG. 4. Unambiguous nonsequential NOEs are consistent with the folding of $[5\text{-}55]_{Ala}$ into a native conformation. (*A*) Section of a NOESY spectrum of $[5\text{-}55]_{Ala}$ in H_2O at 20°C, pH 4.6. A representative set of unambiguous nonsequential NOEs is shown (see Table 2). (*B*) Unambiguous nonsequential NOEs arise from pairs of protons distributed throughout the molecule and are consistent with the formation of both secondary and tertiary native structure. Some of these proton pairs are displayed on a schematic representation (31) of the crystal structure (1–3) for native BPTI (see Table 2).

amino-terminal methionine. A strong correlation between the chemical shifts of the backbone protons in $[5\text{-}55]_{Ala}$ and native BPTI argues (30) that the global fold of these two molecules is similar; in Fig. 3*C* a line with a slope of 1 is shown, representing what would be a perfect correlation.

NOE data support the conclusion that $[5\text{-}55]_{Ala}$ folds into essentially the same conformation as native BPTI (Fig. 4*A*). Thirty-four unambiguous nonsequential NOEs have been identified in spectra of $[5\text{-}55]_{Ala}$. These NOEs arise from pairs of protons that are within 5.4 Å of one another in the crystal structure (1) of native BPTI (Table 2). The three NOEs that arise from protons that are farther apart than 4.6 Å in the crystal structure of native BPTI involve protons at the interface of the α-helix and the β-sheet (Table 2; Fig. 4*B*). This observation is consistent with a minor shift of the α-helix toward the β-sheet, which is observed in the crystal structure (11) of the mutant C30A/C51A, a model for the native intermediate N* (Fig. 1*B*). We conclude that all identified NOEs are consistent with the formation of a native fold in $[5\text{-}55]_{Ala}$ (Fig. 4*B*).

Table 2. Selected NOEs from $[5\text{-}55]_{Ala}$

Structure	NOE proton pair	Distance in native BPTI, Å
α-Helix	48α–51β*	2.3
	50α–53β*	2.7
3_{10}-Helix	3α–6N*	3.7
	4α–6N	3.6
β-Sheet	18N–35N*	3.0
	21δ–32α	3.9
	21α–33δ*	4.6
	21N–45N*	2.8
Tertiary	4ε–45ζ	2.3
	4ε–43α*	3.3
	5β–23ε*	3.7
	6δ–23ε	3.7
	10δ–40α*	3.2
	11α–33δ	3.5
	11α–36N*	3.0
	21δ–48β	4.7
	21δ–51β	4.7
	23N–51β*	5.4
	35ε–40α	2.5
	45δ–51β	2.9

Proton pairs yielding strong, unambiguous NOEs are listed according to their context in the crystal structure (1) of native BPTI. The distance between protons in this context is shown for comparison.
*These pairs are depicted in Fig. 4*B*.

DISCUSSION

Explanation for the Properties of [5-55] in the Folding Pathway of BPTI. Our results indicate that $[5\text{-}55]_{Ala}$ folds into essentially the same conformation as native BPTI. This observation provides an explanation for many of the thermodynamic and kinetic properties of [5-55] in the folding pathway of BPTI as discussed below.

(*i*) Of the 15 possible one-disulfide intermediates, only the intermediates [5-55] and [30-51] accumulate to a significant extent (7) at neutral pH (Fig. 1*B*). The accumulation of [5-55] during folding is explained by the essentially native fold of this intermediate.

(*ii*) At neutral pH, [5-55] forms the 14-38 disulfide bond as fast as the native intermediate N^{SH}_{SH} (Fig. 1*B*) forms the same disulfide bond (ref. 7 and J. S. Weissman, P.S.K., unpublished results). Native structure in [5-55] explains the rapid rate at which it forms the 14-38 disulfide bond (Fig. 1*A*).

(*iii*) The intermediate [5-55] does not readily form the 30-51 disulfide bond (7, 13, 32) (Fig. 1*B*). Native structure in [5-55] should bring Cys-30 and Cys-51 close together, but native structure should also bury these residues (Fig. 1*A*), rendering them inaccessible to external oxidizing agent and thus inhibiting the formation of the 30-51 disulfide bond (see also ref. 39).

(*iv*) Though the intermediate [5-55] predominates over [30-51] at neutral pH, the relative amount of [5-55] is decreased substantially (7) at pH 8.7. The burial of Cys-30 and

Cys-51 (Fig. 1*A*) in the folding of [5-55] will be unfavorable at pH 8.7, where approximately half of all cysteines will be negatively charged. The lower sensitivity of [30-51] to moderately alkaline pH suggests that the burial of cysteines in [30-51] occurs to a lesser degree than in [5-55].

(*v*) At neutral pH, the rearrangement of [5-55] to [30-51] by thiol-disulfide exchange is slow (7) (Fig. 1*B*) relative to the rearrangement of an unfolded one-disulfide intermediate. Native structure in [5-55] will bury the 5-55 disulfide bond (Fig. 1*A*) and restrict the flexibility of the remaining cysteines. Both of these effects will reduce the ability of [5-55] to rearrange.

Implications for the Folding Pathway of BPTI. Protein folding intermediates have been difficult to isolate and characterize structurally (15, 16). The disulfide-bonded intermediates that accumulate in the oxidative folding pathway of BPTI have been trapped successfully (7, 13). Earlier attempts to characterize the structural details of these disulfide-bonded intermediates (33) were hampered by charged carboxymethyl blocking groups that were added to free cysteines in the trapping of these intermediates.

Trapping the one-disulfide intermediate [5-55] by mutating free cysteines to alanine has facilitated the structural and functional characterization of this intermediate. We conclude that [5-55] is not a protein folding intermediate in a structural sense. Moreover, we demonstrate that BPTI requires only a single disulfide bond for folding and function.

Presently, four of the five well-populated disulfide-bonded intermediates in the folding pathway of BPTI have been shown to be essentially completely folded (refs. 9–12; A. Kossiakoff, personal communication). Still, it remains to be seen if the disulfide-bonded intermediate [30-51] (Fig. 1*B*) is a folding intermediate in a structural sense. Studies of a peptide model have shown that a native-like subdomain can form in the presence of the 30-51 disulfide bond (34), and preliminary studies of a recombinant model suggest that [30-51] contains substantial unfolded regions (J.P.S., D. M. Nguyen, and P.S.K., unpublished results).

In designing the recombinant model $[5\text{-}55]_{Ala}$, we aimed to mimic the intermediate [5-55] as it accumulates at neutral pH. During folding under more alkaline conditions (13), at pH 8.7, which is near the pK_a of a cysteine thiol, the stability and structure of the disulfide-bonded intermediates may be perturbed by charged cysteines, as four of the six cysteine sulfurs are buried in native BPTI (Fig. 1*A*). Moreover, since both the charged and neutral forms of cysteine are present at pH 8.7, it is unclear how to model cysteine at this pH with a single mutation. At neutral pH, however, alanine seems to be an appropriate substitution, as alanine, in contrast to serine, will not resist native folding events that may tend to bury free cysteines. The validity of alanine as a substitution is supported by the observation that $[5\text{-}55]_{Ala}$ is predominantly folded at 25°C, a temperature at which [5-55] accumulates significantly during folding at neutral pH; in contrast, a model of [5-55] in which cysteine is replaced with serine is predominantly unfolded at this temperature (18).

Given that $[5\text{-}55]_{Ala}$ is a completely folded protein, we note that it is an attractive model system for studying protein folding. The folding of $[5\text{-}55]_{Ala}$ is coupled to the formation of just a single disulfide bond; this coupling provides a unique opportunity to study global protein stability by measuring the equilibrium (35) between the oxidized and reduced states of $[5\text{-}55]_{Ala}$. The proton NMR assignments of $[5\text{-}55]_{Ala}$, in addition to its stability and solubility, will facilitate further NMR experiments such as hydrogen exchange measurements at equilibrium (36) or during folding (37, 38). The peptide model PαPγ, a subdomain of [5-55], folds into a compact, native-like structure (17). This knowledge, in addition to the wealth of information already known about BPTI, will supplement further studies of $[5\text{-}55]_{Ala}$.

Note Added in Proof. We have investigated why the recombinant model of [5-55] produced by Darby *et al.* and referred to (40) as $[5\text{-}55]_{Ser}$ is substantially less stable than $[5\text{-}55]_{Ala}$. The melting temperature of $[5\text{-}55]_{Ser}$, which contains serine in place of cysteine and the substitution Met-52 → Arg, has recently been reported (40) to be ≈3°C, roughly 35°C less stable than $[5\text{-}55]_{Ala}$. We have found that the substitution Met-52 → Arg in $[5\text{-}55]_{Ala}$ reduces the melting temperature by ≈10°C, as determined by circular dichroism (M. G. Milla, J.P.S., and P.S.K., unpublished results). In contrast, the removal of the amino-terminal methionine in $[5\text{-}55]_{Ala}$ increases the melting temperature by only ≈2°C (M. G. Milla, J.P.S., and P.S.K., unpublished results). Thus, serine substitutions appear to account for most of the instability of $[5\text{-}55]_{Ser}$, although the destabilizing effect of the Met-52 → Arg substitution is significant. Despite the instability of $[5\text{-}55]_{Ser}$, it has been shown recently that this model can also fold into a conformation that is similar to native BPTI (40).

We thank A. Frankel and D. Doering for advice with molecular biology, Z.-Y. Peng and C. J. McKnight for help with NMR experiments, T. Oas and P. Harbury for help with calculations, and J. Weissman for many helpful discussions. J.P.S. is a Howard Hughes Medical Institute Predoctoral Fellow. This research was supported by grants from the Pew Memorial Trust, the Rita Allen Foundation, and the National Institutes of Health (GM41307 and RR05927).

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