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The partially folded conformation of the Cys-30 Cys-51 intermediate in the disulfide folding pathway of bovine pancreatic trypsin inhibitor

(protein folding/NMR/disulfide bonds/folding intermediates)

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ABSTRACT **The best-characterized protein folding pathway is that of bovine pancreatic trypsin inhibitor, which folds from the reduced form through a series of disulfide bond intermediates. The crucial one-disulfide intermediate of bovine pancreatic trypsin inhibitor with the disulfide bond between Cys-30 and Cys-51 is shown here to have a partially folded conformation in which the major elements of secondary structure interact via a core of apolar side chains, which resembles part of the native conformation. The stability of this structure can account for the predominance of this one-disulfide intermediate during folding. Much of the remaining one-third of the polypeptide chain, in particular the N-terminal 14 residues, is largely disordered; this accounts for the ability of this intermediate to form readily any of the three possible second disulfide bonds involving Cys-5, -14, and -38. The partially folded conformation of this intermediate provides direct evidence for the importance of native-like interactions between elements of secondary structure in directing protein folding, which is assumed in many studies.**

The best-characterized folding pathway of a small protein is that of bovine pancreatic trypsin inhibitor (BPTI), which was elucidated by using the relative tendencies of its six Cys residues to form disulfide bonds (1, 2). The most productive folding pathway has been found to be (3, 4)

$$
R \rightleftharpoons \left\{ \begin{matrix} \text{Others} \\ + \\ [30\text{-}51] \end{matrix} \right. \rightleftharpoons \begin{pmatrix} [30\text{-}51;\ 5\text{-}38] \\ + \\ [30\text{-}51;\ 5\text{-}14] \end{pmatrix} \rightleftharpoons [30\text{-}51;\ 5\text{-}55]_N \rightleftharpoons \begin{bmatrix} 30\text{-}51 \\ 5\text{-}55 \\ 14\text{-}38 \end{bmatrix}_N
$$

$$
[30\text{-}51] \searrow [30\text{-}51;\ 14\text{-}38]_N \rightleftharpoons \begin{pmatrix} [30\text{-}51;\ 5\text{-}38] \\ + \\ [30\text{-}51;\ 5\text{-}14] \end{pmatrix}, \qquad \begin{bmatrix} 30\text{-}51 \\ 5\text{-}55 \\ 14\text{-}38 \end{bmatrix}_N = N
$$

Scheme I

The reduced protein with no disulfides is designated R, and the various disulfide-bonded intermediates are designated by the numbers of the Cys residues involved in the disulfides. Intermediates [30-51; 5-14] and [30-51; 5-38] have comparable kinetic roles and are depicted together. Intermediates with the subscript N have essentially fully folded native-like conformations (5, 6). The nonproductive pathway via $[5\text{-}55]_N$ to $[5\text{-}55;\ 14\text{-}38]_N$ has been omitted for simplicity, as disulfide formation in these quasi-native species is primarily controlled by Cys thiol group accessibility and is not linked to protein folding (7–9).

Reduced BPTI is almost completely unfolded and consequently forms one-disulfide intermediates nearly randomly, as depicted by the { in Scheme I. The initial disulfide is interchanged rapidly, and an equilibrium mixture results in which [30-51] predominates. All subsequent intermediates on the productive pathway contain the 30-51 disulfide. A detailed description of the conformational properties of intermediate [30-51] is therefore central to understanding the BPTI folding pathway.

Previous studies using circular dichroism and one-dimensional ^{1}H NMR on the trapped [30-51] (10–12) and on an analogue of it prepared by protein engineering methods (13) suggested that this intermediate contains elements of regular structure and some tertiary interactions. In particular, the initial one-dimensional NMR studies on the trapped intermediates (12) suggested the presence of a native-like central β-sheet in [30-51]. A peptide model of [30-51] was proposed to contain elements of α-helix and β-sheet like those found in fully folded BPTI (14). This model, $P_\alpha P_\beta$, comprised two peptides corresponding to residues 20 to 33 and 43 to 58 linked by a disulfide bond between Cys-30 and Cys-51. To characterize the folding intermediates, and in particular [30-51], in greater detail, analogues have been prepared by protein engineering in which the Cys residues not involved in disulfide bonds are replaced by Ser residues, which differ only in the sulfur atom being replaced by an oxygen atom (6, 9, 13, 15). These analogues avoid the potential difficulties of the blocking groups that are introduced in trapping the intermediates and have the other advantage that any intermediate can be made in substantial quantities and in homogeneous form.

The nature of the nonrandom conformation in [30-51] is important to account for its predominance among the one-disulfide intermediates, and it is also necessary to explain the subsequent steps in the folding pathway. Intermediate [30-51] does not readily form the 5-55 disulfide bond directly but instead forms a second disulfide between Cys-5 and -14, Cys-5 and -38, or Cys-14 and -38. Formation of the nonnative intermediates [30-51; 5-14] and [30-51; 5-38] directly from [30-51] was concluded to be the most productive path of folding (16). This aspect of the pathway has recently been challenged, with the proposal of a revised BPTI folding pathway (17):

$$
R \rightleftharpoons [30\text{-}51] \rightleftharpoons [30\text{-}51;\ 14\text{-}38] \rightleftharpoons [30\text{-}51;\ 5\text{-}55] \rightleftharpoons N
$$

Scheme II

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Abbreviations: BPTI, bovine pancreatic trypsin inhibitor; [Arg^{52}]BPTI, BPTI produced in *Escherichia coli* with Met-52 changed to Arg; [30-51], etc., the normal disulfide-bonded intermediates that are trapped during folding are designated by the residue numbers of the Cys residues paired in disulfide bonds; $[30\text{-}51]_{\text{Ser}}$, [Arg^{52}]BPTI with a single disulfide bond, between Cys-30 and Cys-51, and with the Cys-5, Cys-14, Cys-38, and Cys-55 residues changed to Ser; NOE, nuclear Overhauser enhancement; NOESY, NOE spectroscopy; ROESY, rotating-frame NOESY; TOCSY, total correlated spectroscopy; 2D, two-dimensional.

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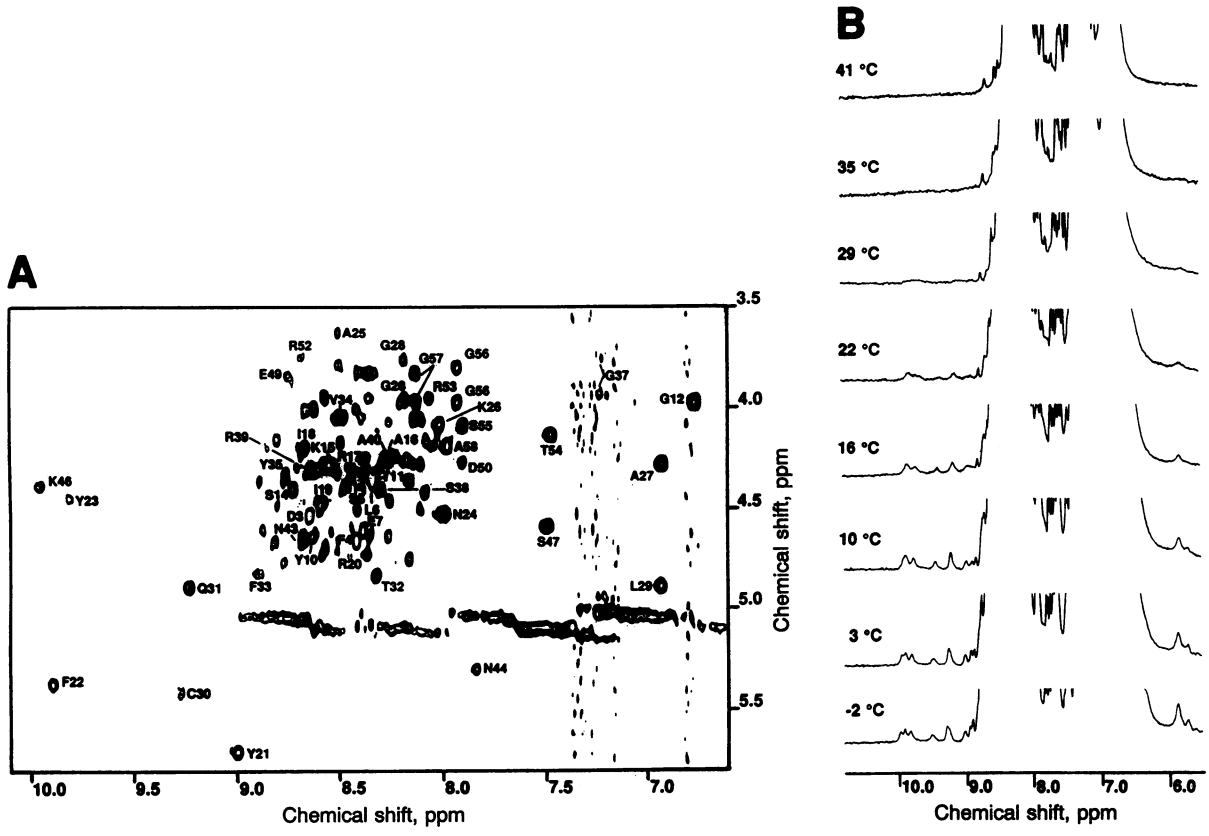

FIG. 1. (*A*) 2D TOCSY spectrum of intermediate $[30\text{-}51]_{Ser}$ at −2°C, with a τ_m of 30 ms. The majority of the cross-peaks from folded $[30\text{-}51]_{Ser}$ have been labeled. Note that many of the resonances are dispersed, as with a folded protein, whereas the others cluster together near the center of the (NH, $C^{\alpha}H$) region, as expected for an unfolded polypeptide. The spectrum also includes many unassigned cross-peaks in the center of the (NH, $C^{\alpha}H$) region, which arise from a small fraction of unfolded molecules. The intensities of these cross-peaks were weak in NOESY spectra. (*B*) Low-field region of the ^1H NMR spectra of $[30\text{-}51]_{Ser}$ at various temperatures. The lines broaden on increasing the temperature, presumably because of exchange between folded and unfolded molecules with an intermediate rate on the chemical shift time scale.

The quasinative [5-55] and [5-55; 14-38] have again not been depicted for simplicity, as in Scheme I. The revised pathway (Scheme II) differs from the original (Scheme I) primarily in that it omits the nonnative [30-51; 5-14] and [30-51; 5-38] intermediates, because they were observed not to be highly populated during folding (17). Nonnative intermediates were proposed (17) to arise only by unfolding and rearrangement of [30-51; 14-38], which is depicted as an obligatory intermediate in folding (Scheme II). It is not necessary for a crucial kinetic intermediate to be highly populated, however, and the experimental kinetic evidence (4, 16) indicates that [30-51; 5-14] and [30-51; 5-38] are important intermediates in folding and are formed most productively directly from [30-51], as in Scheme I. The kinetic data also indicate that intermediate $[30\text{-}51; 14\text{-}38]_N$ is not an obligatory intermediate in folding; its accumulation to high levels is primarily a result of the stability of its quasi-native conformation (6).

We report here the results of a two-dimensional ^1H NMR study of an analogue of intermediate [30-51], which explain why this intermediate plays such a crucial role in BPTI folding. In particular, the reasons for its stability and the ease with which it forms the nonnative 5-14 and 5-38 disulfide bonds can now be understood. In accord with our general convention for naming such analogues of BPTI folding intermediates, this analogue is designated $[30\text{-}51]_{Ser}$; the disulfide bonds present in the intermediate appear within the bracket, and the subscript Ser indicates that all other cysteine residues, in this case 5, 14, 38, and 55, are changed to serine residues.

METHODS

Protein Expression and Purification. The proteins were expressed in *Escherichia coli* in the reduced form, purified, oxidized to form the appropriate disulfide bonds, and purified to homogeneity by the method used previously (ref. 15; N.J.D. and T.E.C., unpublished results). Cyanogen bromide was used to cleave the BPTI sequence out of a fusion protein, so the normal Met-52 residue of BPTI has been replaced by Arg, which is the residue most frequently found at this position in the homologues of BPTI (18). All data on $[30\text{-}51]_{Ser}$ are compared with data obtained with an analogue of the three-disulfide native protein that also contains this mutation, $[Arg^{52}]$BPTI, and is very similar to normal BPTI (6).

NMR Analysis. NMR data were collected by using a Bruker 500-MHz AMX spectrometer. Samples of $[30\text{-}51]_{Ser}$ and $[Arg^{52}]$BPTI contained approximately 2 to 3 mM protein at pH 4.6 in either 90% ^1H_2O/10% ^2H_2O (vol/vol) or 100% ^2H_2O. Data acquisition times ranged from 18 to 44 hr, with 4096 time-domain points in 512 increments. Except in the jump-return nuclear Overhauser enhancement (NOE) spectroscopy (NOESY) experiments, water suppression was achieved by selective presaturation during the relaxation delay (1.5 s). Double-quantum filtered correlated spectroscopy (DQF-COSY), double-quantum spectroscopy (DQ) (mixing time τ_m = 40, 50 ms), rotating-frame NOESY (ROESY) (τ_m = 30 ms), total correlated spectroscopy (TOCSY) (τ_m = 30–60 ms), NOESY (τ_m = 100 ms), and NOESY experiments with a jump return read sequence ($45°_{\phi}$–80-μs delay–$45°_{\phi+180°}$) (τ_m = 150 ms) were used for spin system and sequential assignments. Two-dimensional (2D) spectra were acquired at temperatures of −2, 3, 10, 16, and 22°C. The 2D NMR data were digitally filtered after acquisition by using a sine-bell shifted by 22.5° in the t_2 dimension and a squared sine-bell shifted by 30° (and 90° in the case of DQF-COSY and DQ) in the t_1 dimension. After double

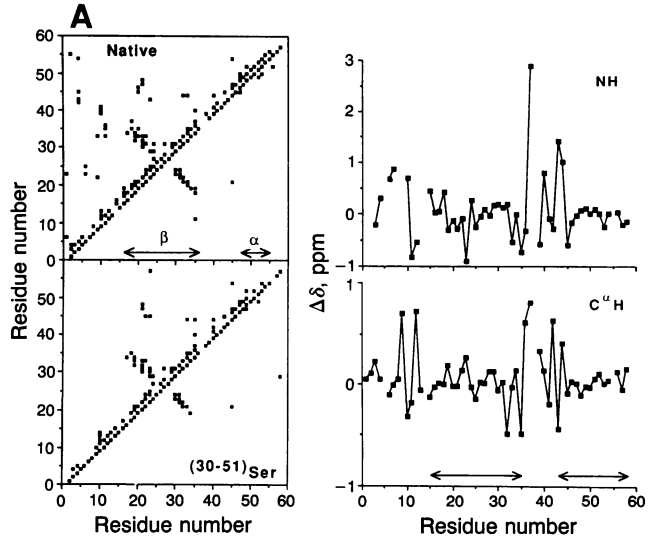

FIG. 2. Structural analysis of $[30\text{-}51]_{Ser}$. (*A*) Comparison of the observed NOE contacts between amino acid residues within $[30\text{-}51]_{Ser}$ at −2°C (*Lower*) and the corresponding analogue of native BPTI at 10°C (*Upper*). In each plot a point below the diagonal indicates an NOE connectivity between NH and NH, NH and $C^{\alpha}H$, or $C^{\alpha}H$ and $C^{\alpha}H$ protons (or $C^{\alpha}H$ and $C^{\delta}H$ when proline residues are involved). A point above the diagonal indicates connectivities between a backbone and a side-chain proton or between side-chain and side-chain protons. The NOE contacts were obtained from 100-ms NOESY spectra. The α-helix and β-sheet elements of secondary structure in native BPTI are indicated. (*B*) Differences in chemical shift position (Δδ) between $[30\text{-}51]_{Ser}$ at −2°C and the corresponding analogue of native BPTI at 10°C for the NH (*Upper*) and $C^{\alpha}H$ (*Lower*) protons, plotted versus amino acid residue number. The horizontal arrows indicate the residues that have a folded conformation in $[30\text{-}51]_{Ser}$ like that in the native conformation. The resonances were assigned in the usual manner (19) from spectra acquired at low temperatures. Residues omitted are those that differ chemically (i.e., Ser in place of Cys) or have not been assigned.

Fourier transformation of the data, a third-order polynomial baseline correction was applied in the F_2 dimension to all spectra. A one-dimensional (1D) version of the three-pulse NOESY pulse sequence and phase cycle was used to collect the 1D NMR data; the value of the incremental delay t_1 was fixed at 3 μs and τ_m was kept very short (5 ms). No window functions were applied to the 1D NMR data, and no zero filling was applied.

RESULTS

$[30\text{-}51]_{Ser}$ gave NMR spectra at low temperatures (Fig. 1) that suggest that part of the polypeptide chain has a relatively ordered structure, while the remainder is largely disordered. The protons of the structured residues had widely dispersed chemical shifts and NOE contacts to residues distant in the primary structure. The resonances of many of the backbone amide protons of these residues were broadened to various extents when compared with their counterparts in native BPTI. In contrast, protons of the disordered residues had sharp resonances with chemical shifts close to their random coil values (19), strong $d_{\alpha N}$ NOE contacts, and sharp TOCSY cross-peaks connecting many of the backbone amide protons to their corresponding side-chain signals.

The 2D NMR spectra also included cross-peaks in the center of the (NH, $C^{\alpha}H$) region that could not be assigned. These cross-peaks presumably arise from a small fraction of molecules that are unfolded. The intensities of these cross-peaks were low in NOESY spectra, but TOCSY experiments emphasize the sharper lines in a spectrum, so that cross-peaks from unfolded molecules are disproportionately intense (9). The resonances from the folded structure shifted and broadened as the temperature was increased, especially in the range 16–29°C, where they also decreased in intensity (Fig. 1*B*). In contrast, resonances from the unfolded portion shifted, but remained sharp, when the temperature was increased.

The NMR spectra were assigned at −2°C, not to maximize the population of the folded state but to minimize the extent of broadening of the signals due to intermediate rate exchange between folded and unfolded molecules (cf. ref. 9). The broadening of the resonances prevented the use of long mixing times in TOCSY experiments, which complicated the assignment process; nonetheless, the sequential assignments could be made by following the usual strategy (19) and have been confirmed by using a ^{15}N-labeled sample of $[30\text{-}51]_{Ser}$ in heteronuclear 2D NMR experiments.

The folded portions of $[30\text{-}51]_{Ser}$ were found to consist of residues 15–36 and approximately from residue 43 to residue 58. Fig. 2*A* shows that the pattern of NOE connectivities in these regions of $[30\text{-}51]_{Ser}$ is very similar to the corresponding analogue of native BPTI ($[Arg^{52}]$BPTI), which was shown (6) to have a conformation indistinguishable from normal BPTI. Also, protons in these regions had chemical shifts (Fig. 2*B*) that do not differ markedly from those in $[Arg^{52}]$BPTI; 81% of all the assigned resonances for the folded portion of $[30\text{-}51]_{Ser}$ exhibited chemical shift differences of ≤0.20 ppm. Fig. 3 shows NOESY spectra demonstrating part of the assignment pathway for the C-terminal α-helix and central β-sheet. The β-sheet is also apparent from the stripe perpendicular to the diagonal in the NOE contact map (Fig. 2*A*). The packing of the α-helix and β-sheet appears to be the same as in the native structure of BPTI, where they interact through apolar side chains. Several small NOE and chemical shift (Fig. 2*A*) differences, however, indicate that the folded portion of $[30\text{-}51]_{Ser}$ is not exactly like that in native BPTI. The absence of the Gly-56 NH to Arg-52 $C^{\alpha}H$ NOE cross-peak indicates that the C-terminal hydrogen bond of the α-helix is probably absent. The conformational difference at the C terminus of the polypeptide chain is confirmed by a new NOE contact between Ala-58 NH and Leu-29 $C^{\alpha}H$ in the intermediate analogue. Further small differences in NOE contacts indicate that the ends of the β-strands are probably somewhat distorted and that the orientations of the side chains of Arg-17, Tyr-21, and Phe-45, and possibly Tyr-23 and Tyr-35, are somewhat different from those in native BPTI.

Of the remainder of the $[30\text{-}51]_{Ser}$ molecule, the N-terminal 14 residues in particular appear to be largely disordered, making no NOE contacts with the folded core of the molecule, in contrast to native BPTI (Fig. 2*A*) (6). On the other hand, some indications of local nonrandom conformation in residues 10–12 were observed, for example, by the chemical shift of the NH of Gly-12 not being that expected for a random-coil backbone amide proton (Fig. 1*A*). The conformations of residues 35–42 resemble those in native BPTI with some differences and probably an increased flexibility. This is suggested by differences in NOE contacts of residues 37, 38, and 39, decreased linewidths of the amide resonances of residues 38, 39, and 40, and an increased ring flip rate of Tyr-35.

At low temperatures weak cross-peaks appear in the low-field region of TOCSY, NOESY, and ROESY spectra, connecting signals of the folded protein with cross-peaks that could not be assigned. These weak cross-peaks must be due to chemical exchange between folded and (presumably) unfolded molecules that is fast on the NMR T_1 time scale but not on the chemical shift time scale ($T_1^{-1} < k_{exch} < \Delta\delta$).

The ^{15}N relaxation properties of ^{15}N-labeled $[30\text{-}51]_{Ser}$ have also been studied. Preliminary results concerning the $^{15}N\{^1H\}$NOE enhancements strongly support the results dis-

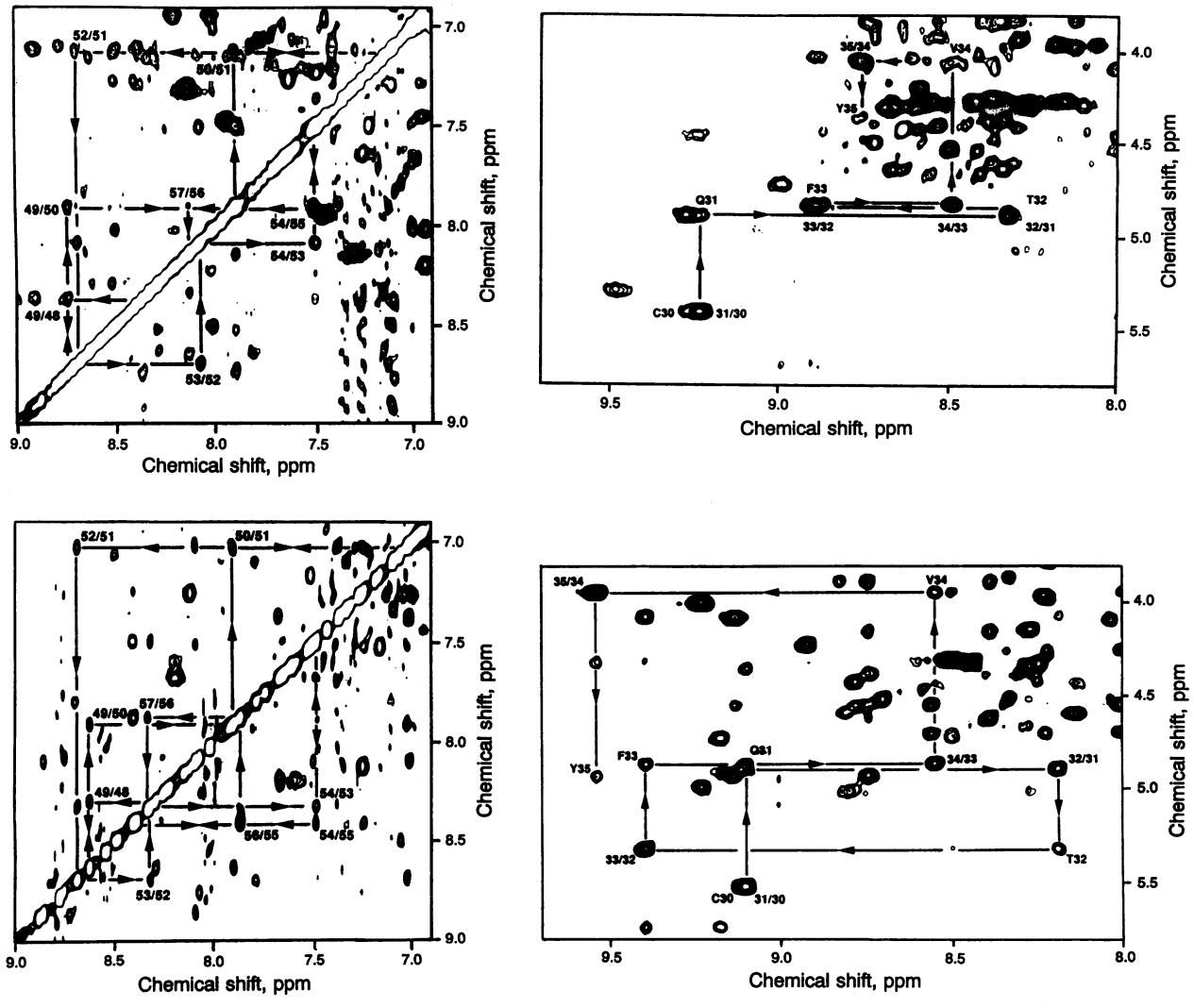

FIG. 3. Sequential assignment pathway for part of the C-terminal α-helix (*Left*) and of the β-sheet (*Right*) of both $[30\text{-}51]_{Ser}$ (*Upper*) at −2°C and the analogue of native BPTI (*Lower*) at 10°C, illustrated on parts of NOESY spectra (τ_m = 100 ms, *Left*; τ_m = 150 ms, *Right*). Inter-residue cross-peaks are labeled F_2/F_1.

cussed here, in that the N terminus of the molecule exhibits markedly more negative NOE enhancements, reflecting a greater degree of local flexibility.

DISCUSSION

The equilibrium conformations of disulfide-trapped intermediates of BPTI, as for $[30\text{-}51]_{Ser}$, are likely to be similar to those of the intermediates formed during folding. Most conformational changes during folding under normal conditions appear to be rapid relative to the rates of disulfide formation and are therefore not rate-limiting (20). This implies that any conformation stabilized by a particular disulfide bond should be stabilized to the same extent by that disulfide bond in the trapped intermediate (21).

The data presented here provide a detailed description of the structure of the [30-51] folding intermediate of BPTI (Fig. 4). The α-helix and β-sheet that constitute the main regular structural elements of the fully folded molecule are shown here to be present also in the intermediate. The way in which these major elements of secondary structure pack together by nonpolar interactions resembles that in native BPTI. This stable partially folded conformation of intermediate [30-51] is probably sufficient to account for its relatively low free energy and consequent predominance amongst the other one-disulfide intermediates. The disorder of part of the $[30\text{-}51]_{Ser}$ molecule, which includes Cys-5, Cys-14, and to some extent also Cys-38, is consistent with this intermediate forming any of the three possible disulfide bonds between

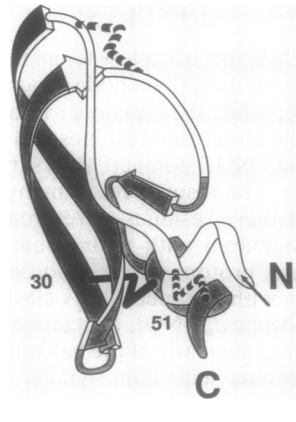

FIG. 4. Schematic drawing of the backbone topology of native BPTI, with the segments that are folded in $[30\text{-}51]_{Ser}$ shaded. The segments that are not shaded are disordered (N terminus) or appear to have an increased flexibility. The 30-51 disulfide bond is indicated in black. The 14-38 and 5-55 disulfides of native BPTI that are absent in $[30\text{-}51]_{Ser}$ are dashed.

these cysteine residues during folding: each of these residues is in a flexible region of $[30\text{-}51]_{Ser}$. The rates of formation of these bonds are comparable to those at which initial disulfides form in fully reduced BPTI. This explains why the 14-38 disulfide is formed at less than 1% of the rate at which it is formed in the native-like [30-51; 5-55] intermediate (3).

Intermediate [30-51] is a true partially folded intermediate in protein folding. The other intermediates in BPTI folding are either very unfolded and show little or no nonrandom conformation, such as R, [5-30], and [5-51] (12, 13), or demonstrate a native-like conformation of the entire molecule at low temperatures that unfolds cooperatively at higher temperatures (6–9). Each of the species with two native disulfide bonds is fully folded and structurally similar to native BPTI with all three disulfides, but less stable, depending upon the degree of stabilization of the individual disulfide bonds. The native conformation of BPTI is stable even at high temperatures or in concentrated denaturants (22) and is therefore still present at room temperature when any one of the native disulfide bonds is absent. It is even somewhat stable with only the single native disulfide 5-55, which is the disulfide that contributes most to the stability of the native conformation. 2D NMR studies show $[5\text{-}55]_{Ser}$ to be fully folded about half the time at 3°C (9), whereas at 25°C and pH 8.7 its rate of forming the 14-38 disulfide (3) indicates that it is native-like only about 10% of the time. In contrast, $[30\text{-}51]_{Ser}$ does not have a quasi-native conformation, even at −2°C. Its partially folded conformation is distinct and populated at temperatures where $[5\text{-}55]_{Ser}$ appears to be fully unfolded (9).

The location and nature of the folded regions in the partially folded conformation of $[30\text{-}51]_{Ser}$ demonstrate the importance of interactions between elements of native-like secondary structure in protein folding, as was also demonstrated by the peptide model $P_\alpha P_\beta$ (14). The results presented here go beyond those reported for the peptide model, from which part of the polypeptide chain was missing; all the parts that were present in $P_\alpha P_\beta$ were folded, so that its structure provided no information about the missing portion of the chain, much of which is flexible in [30-51]. Also, $P_\alpha P_\beta$ could form only a less extensive β-sheet than $[30\text{-}51]_{Ser}$, because residues 15–19 and 34–36 were absent. This may explain why exchange of the folded and unfolded conformations was slower for $[30\text{-}51]_{Ser}$ than for $P_\alpha P_\beta$; on the chemical shift time scale $[30\text{-}51]_{Ser}$ was in the intermediate exchange rate region, whereas the peptide model was in fast exchange (14).

Our results are also relevant to the recently proposed revised pathway for BPTI folding (Scheme II) (17), because they contradict the structural arguments used in its support. In that pathway, the only productive step from [30-51] was formation of the 14-38 disulfide bond to give the quasi-native [30-51; 14-38]. Formation of the nonnative [30-51; 5-14] and [30-51; 5-38] was assumed to occur only by intramolecular rearrangement of quasinative [30-51; 14-38], which would require at least some unfolding. Consequently, the nonnative intermediates were regarded as high-energy, essentially unfolded, nonspecific, transient intermediates. That conclusion neglected the kinetic evidence (16) that intermediates [30-51; 5-14] and [30-51; 5-38] arise most productively from partially folded [30-51]. That evidence, combined with the present results, shows that folding in the original BPTI pathway does not need to be followed by unfolding and refolding (Scheme I). The nonnative 5-14 and 5-38 disulfides can form in flexible regions of the [30-51] structure without unfolding of its folded regions. Indeed, intermediates $[30\text{-}51; 5\text{-}14]_{Ser}$ and $[30\text{-}51; 5\text{-}38]_{Ser}$ have folded conformations similar in extent to that of $[30\text{-}51]_{Ser}$ (ref. 13 and unpublished observations). Whether or not additional nonrandom conformations stabilize the nonnative 5-14 and 5-38 disulfides remains to be determined. If not, the primary roles of these disulfides at the two-disulfide stage may be production of a flexible structure in which Cys-5 and Cys-55 can become joined intramolecularly by disulfide rearrangements leading to the stable fully folded conformation.

Why does the intermediate [30-51] form the 5-55 disulfide bond directly at only 5×10^{-4} the rate at which the other second disulfides are formed (16, 23)? Conceivably the folded conformation of [30-51] could keep Cys-5 and -55 sufficiently far apart to hinder formation of a disulfide bond between them. However, the conformation of the product, [30-51; 5-55], may be more relevant to this question, because it has a fully native conformation in which the 5-55 disulfide bond is buried. According to general observations in several other proteins, buried disulfides are reduced slowly, probably because they are inaccessible to reducing agents; they are also observed to be formed slowly in cases where the product would have a stable folded conformation (1, 9). These observations suggest that the same high-energy transition state is encountered in the reduction of buried disulfide bonds as in the formation of disulfides that will become buried. Similar energetic considerations are likely to apply to formation of buried hydrogen bonds in protein folding (1) but will be smaller in magnitude. The disulfide rearrangements in the folding of BPTI are probably extreme manifestations of the rate-limiting conformational rearrangements along the pathways to the native conformations of other proteins (1).

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