Protein Science (1994), *3*:2226–2232. Cambridge University Press. Printed in the USA.

Hydrogen exchange in BPTI variants that do not share a common disulfide bond

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(RECEIVED July 20, 1994; ACCEPTED September 8, 1994)

Abstract

Bovine pancreatic trypsin inhibitor (BPTI) is stabilized by 3 disulfide bonds, between cysteines 30-51, 5-55, and 14-38. To better understand the influence of disulfide bonds on local protein structure and dynamics, we have measured amide proton exchange rates in 2 folded variants of BPTI, $[5\text{-}55]_{Ala}$ and $[30\text{-}51; 14\text{-}38]_{V5A55}$, which share no common disulfide bonds. These proteins resemble disulfide-bonded intermediates that accumulate in the BPTI folding pathway. Essentially the same amide hydrogens are protected from exchange in both of the BPTI variants studied here as in native BPTI, demonstrating that the variants adopt fully folded, native-like structures in solution. However, the most highly protected amide protons in each variant differ, and are contained within the sequences of previously studied peptide models of related BPTI foldiing intermediates containing either the 5-55 or the 30-51 disulfide bond.

Keywords: amide proton exchange; BPTI; disulfide; NMR; protein dynamics; protein folding; subdomain

Disulfide bonds stabilize substantially the native state of many proteins. This stabilizing effect has been attributed both to the restriction of conformational entropy in the unfolded polypeptide and to enthalpic stabilization of the folded state (for a review, see Betz, 1993). A quantitative understanding of the effects of disulfide bonds on protein stability will therefore require knowledge of the effects of crosslinks on the folded state as well as the unfolded state.

BPTI is a very stable 58-residue protein stabilized by 3 disulfide bonds, between residues 30-51, 5-55, and 14-38 (Fig. 1A). BPTI unfolds spontaneously upon reduction of these disulfide bonds, even in the absence of denaturants. Thus, the folding of BPTI is thermodynamically coupled to disulfide bond formation (Creighton, 1977; Creighton & Goldenberg, 1984; Weissman & Kim, 1991, 1992b).

The folding pathway of BPTI (Fig. 1B) has been studied extensively in terms of the disulfide-bonded intermediates that accumulate during folding (Creighton, 1977; Creighton & Goldenberg, 1984; Weissman & Kim, 1991, 1992b). Two interesting intermediates that accumulate during folding are [5-55] and [30-51; 14-38]. Both of these intermediates have been shown to fold into native-like conformations (Altman et al., 1991; Weissman & Kim, 1991; van Mierlo et al., 1991a, 1991b; Staley & Kim, 1992). Thus, no particular disulfide bond is essential for adopting the BPTI fold.

We focus here on the amide proton exchange properties of 2 completely folded recombinant variants of BPTI: $[5\text{-}55]_{Ala}$, retaining only the 5-55 disulfide bond, with Cys 14, 30, 38, and 51 replaced by Ala (Staley & Kim, 1992); and $[30\text{-}51; 14\text{-}38]_{V5A55}$, containing both the 30-51 and 14-38 disulfide bonds, with Cys 5 replaced by Val and Cys 55 by Ala (Altman et al., 1991). Our amide proton exchange results confirm that both $[5\text{-}55]_{Ala}$ and $[30\text{-}51; 14\text{-}38]_{V5A55}$ adopt native-like structures in solution and indicate that the arrangement of disulfide bonds affects local structural fluctuations in the folded state of BPTI.

Results

NMR assignments of $[30\text{-}51; 14\text{-}38]_{V5A55}$

The high-resolution crystal structure of $[30\text{-}51; 14\text{-}38]_{V5A55}$ is very similar to that of native BPTI (A.A. Kossiakoff, pers. comm.). The similarity in the chemical shifts of the α and amide protons in $[30\text{-}51; 14\text{-}38]_{V5A55}$ and BPTI (Fig. 2A,B) and a number of unambiguous, readily identifiable, nonsequential NOEs (Fig. 2C) indicate that $[30\text{-}51; 14\text{-}38]_{V5A55}$ also folds in solution into a conformation very similar to native BPTI.

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Abbreviations: BPTI, bovine pancreatic trypsin inhibitor; H-D, hydrogen–deuterium; HSMQC, heteronuclear single-multiple quantum coherence; HSQC, heteronuclear single quantum coherence; NOESY, nuclear Overhauser effect spectroscopy; 1D, 1-dimensional; TOCSY, total correlation spectroscopy.

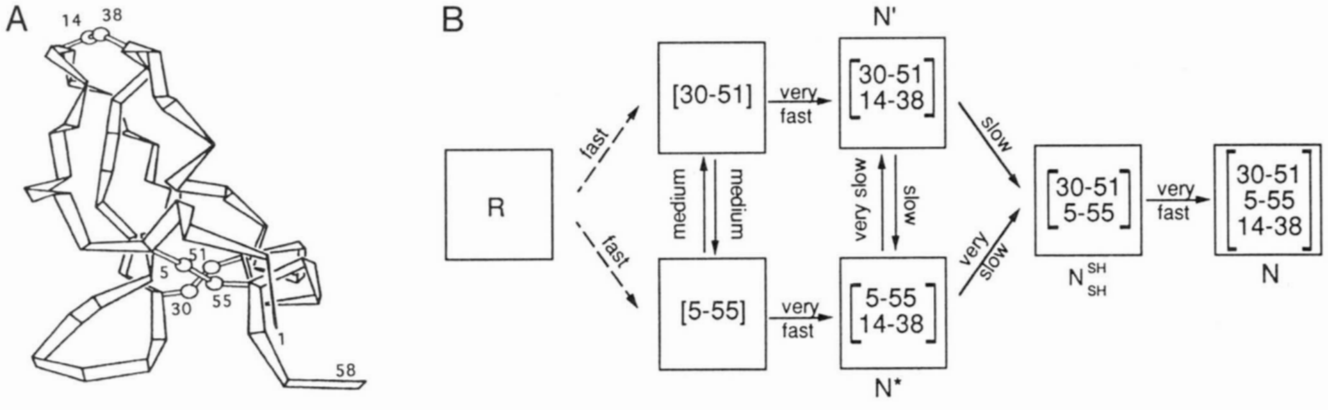

Fig. 1. A: Schematic representation (adapted from Creighton, 1975) of the crystal structure of BPTI (Deisenhofer & Steigemann, 1975; Wlodawer et al., 1984, 1987), with labels for the cysteine residues involved in disulfide bonds and the first and last residues. The disulfide bond 14-38 is accessible to solvent, exposing 50% of its total surface area, whereas the disulfide bonds 30-51 and 5-55 are inaccessible, exposing 0% of their total surface area (Lee & Richards, 1971). **B:** Schematic diagram of the folding pathway of BPTI at 25 °C, pH 7.3 (Weissman & Kim, 1991, 1992b). 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 folding of N* can be accelerated ~6,000-fold by protein disulfide isomerase (Weissman & Kim, 1993). The dashed arrows indicate that the major 1-disulfide intermediates do not form directly from reduced protein but rather from rearrangement of other 1-disulfide intermediates (Creighton, 1977). The relative rates of intramolecular transitions at pH 7.3 are indicated; "very fast" rates are on the order of milliseconds, whereas "very slow" rates are on the order of weeks (Weissman & Kim, 1992c). N^{SH}_{SH}, N*, [30-51; 14-38], and [5-55] fold into essentially the same conformation as native BPTI (Stassinopoulou et al., 1984; States et al., 1984, 1987; Eigenbrot et al., 1990, 1992; Altman et al., 1991; van Mierlo et al., 1991a, 1991b; Hurle et al., 1992; Staley & Kim, 1992; A.A. Kossiakoff, pers. comm.).

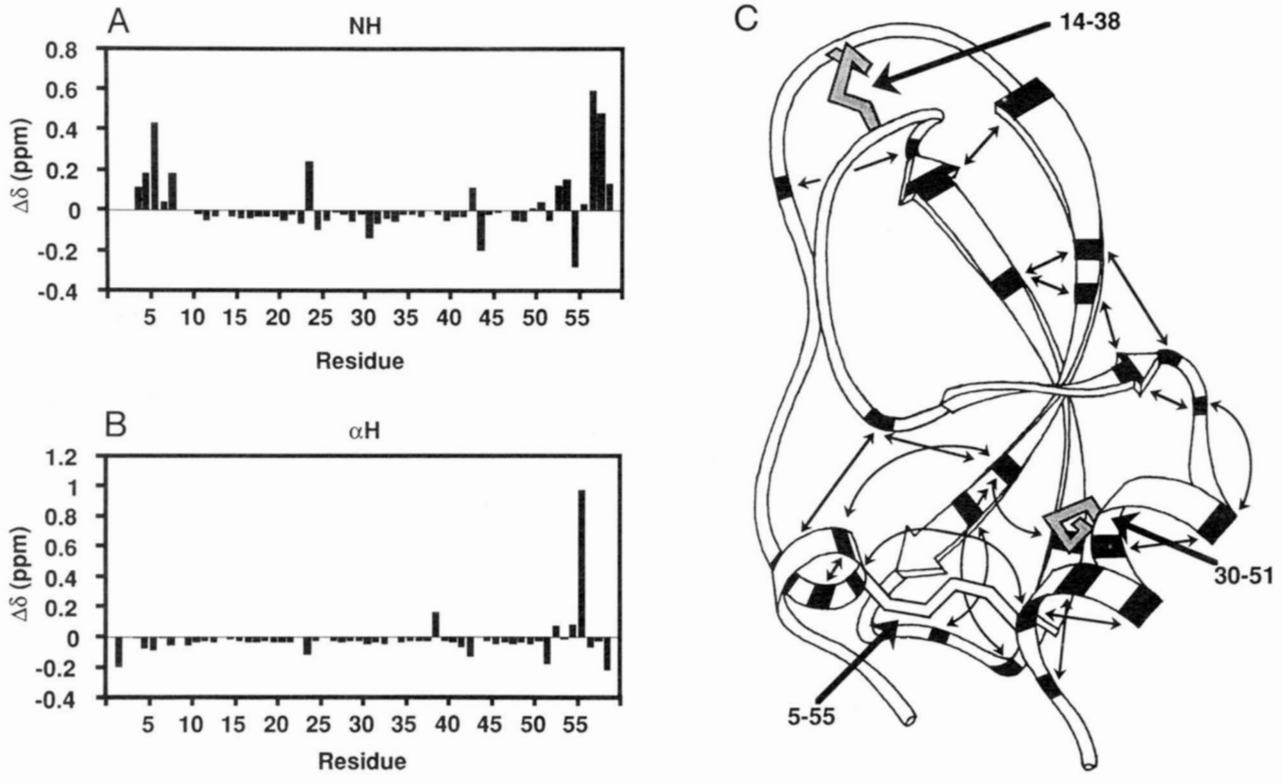

Fig. 2. Differences in chemical shift ($\Delta\delta$) between native BPTI (Staley, 1993) and [30-51; 14-38]$_{V5A55}$ at pH 4.6, 20 °C for the (**A**) amide and (**B**) α protons, plotted versus amino acid residue number. A positive $\Delta\delta$ value represents an upfield-shifted proton in [30-51; 14-38]$_{V5A55}$ as compared to native BPTI. **C:** Unambiguous nonsequential NOEs arise from pairs of protons distributed throughout [30-51; 14-38]$_{V5A55}$ and are consistent with the formation of native secondary and tertiary structure. Some of these proton pairs are displayed on a schematic representation (Richardson, 1985; Staley & Kim, 1992) for native BPTI.

Aside from resonances from the mutated residues, only 5 α or amide proton chemical shifts differ by greater than 0.2 ppm between [30-51; 14-38]$_{V5A55}$ and BPTI, and all of these protons are close to sites of mutation. These 5 protons arise from residues that include Tyr 23, Thr 54, and Gly 56, which are within 6 Å of the 5-55 disulfide bond in native BPTI (Wlodawer et al., 1984). The other 2 residues with large changes in chemical shift are Gly 57 and Ala 58; these differences may result from the addition of a methionine residue to the N-terminus of [30-51; 14-38]$_{V5A55}$, potentially interfering with a hydrogen bonded salt bridge between the α-carboxylate of Ala 58 and the free amino-terminus of Arg 1 in native BPTI (Brown et al., 1978).

Amide proton exchange

The rate constants of exchange at pH 4.6 for amide protons in [5-55]$_{Ala}$ and [30-51; 14-38]$_{V5A55}$ were measured at 5 °C and 20 °C, and additionally at 30 °C for [30-51; 14-38]$_{V5A55}$ (Fig. 3; Table 1). Both [5-55]$_{Ala}$ and [30-51; 14-38]$_{V5A55}$ are monomeric in exchange buffer at 5 °C as determined by equilibrium sedimentation (see Materials and methods). The patterns of protection from amide proton exchange (Fig. 4) in [5-55]$_{Ala}$ and [30-51; 14-38]$_{V5A55}$ are very similar to each other and to BPTI (Wagner & Wüthrich, 1982a; Wagner et al., 1984). All of the amide hydrogens involved in regular secondary structure in BPTI are significantly protected in both disulfide variants.

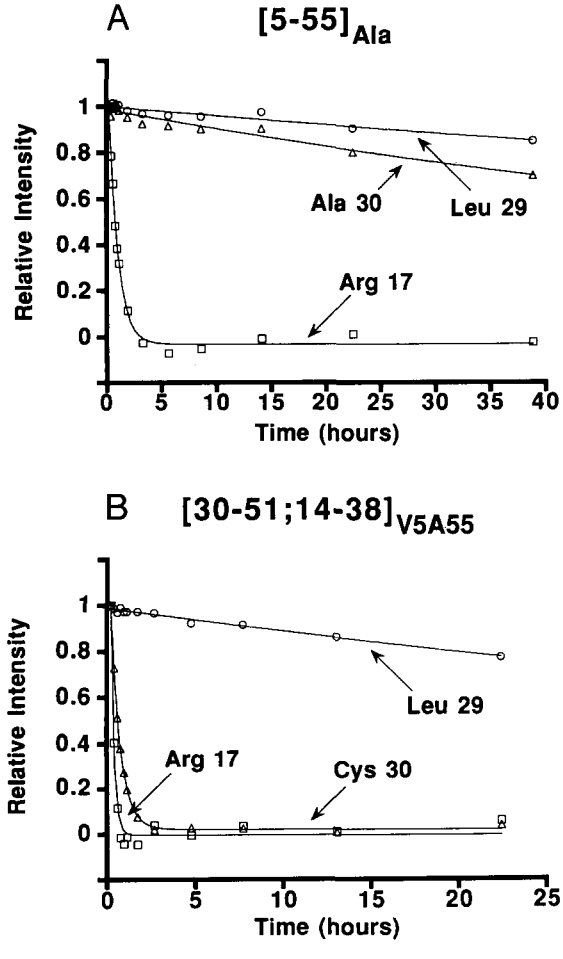

Fig. 3. The decay in intensity of the ^{15}N-^{1}H HSQC correlation for the amide protons of residues 17 (squares), 29 (circles), and 30 (triangles) in (**A**) [5-55]$_{Ala}$ at 5 °C, pH* 4.6; and (**B**) [30-51; 14-38]$_{V5A55}$ at 20 °C, pH* 4.6.

Table 1. *Amide proton exchange rates ($\times 10^{-4}$ min^{-1}) of BPTI variants at pH* 4.6*

		[30-51; 14-38]$_{V5A55}$			[5-55]$_{Ala}$	
Residue[a]	H bonded[b]	5 °C	20 °C	30 °C	5 °C	20 °C
Cys-5 (Val)	+	12	96	390	1.1	72
Leu-6	+	14	110	430	0.70	40
Glu-7	+	10	84	290	0.36	23
Tyr-10		2.6	24	140	4.3	160
Gly-12		26	220	930	13	390
Cys-14 (Ala)		10	1,300	1,100	350	Fast
Ala-16	+	1.6	22	200	18	440
Arg-17		140	900	5,900	190	Fast
Ile-18	+	0.16	2.6	25	1.1	60
Ile-19		7.6	52	240	4.5	89
Arg-20	+	0.094	1.9	25	1.1	99
Tyr-21	+	0.076	1.2	17	0.96	88
Phe-22	+	0.22	1.5	20	1.1	95
Tyr-23	+	0.14	3.2	35	1.4	110
Asn-24	+	0.16	4.8	56	3.0	250
Ala-25		74	570	2,800	13	480
Lys-26	+	400	Fast	Fast	230	Fast
Ala-27	+	13	83	330	8.6	190
Gly-28	+	1.8	13	84	2.5	190
Leu-29		0.096	2.2	24	1.1	91
Cys-30 (Ala)		35	3,100	1,300	2.1	120
Gln-31	+	0.20	2.8	34	1.5	150
Thr-32		3.1	25	160	3.8	250
Phe-33	+	0.068	1.5	18	1.5	130
Val-34		3.4	25	110	2.7	90
Tyr-35	+	0.29	4.2	37	1.1	92
Gly-36	+	3.8	42	370	6.2	460
Gly-37		1.9	28	290	7.9	560
Cys-38 (Ala)	+	15	180	1,400	97	Fast
Ala-40		340	Fast	Fast	190	Fast
Lys-41		8.9	110	870	6.5	520
Arg-42		230	Fast	Fast	120	Fast
Asn-43	+	55	620	6,500	18	700
Asn-44	+	2.3	45	500	6.4	660
Phe-45	+	0.60	14	170	2.9	260
Ser-47		17	110	500	4.3	260
Ala-48		110	790	630	20	630
Glu-49		500	Fast	Fast	130	2,100
Asp-50	+	210	1,600	5,400	19	370
Cys-51 (Ala)	+	0.32	5.2	63	0.78	63
Met-52	+	0.28	3.4	36	0.58	43
Arg-53	+	0.42	5.9	61	0.86	71
Thr-54	+	5.6	39	200	1.9	130
Cys-55 (Ala)	+	6.9	46	250	2.3	210
Gly-56	+	19	150	680	2.9	210
Gly-57		430	Fast	Fast	290	Fast
Ala-58		Fast	Fast	Fast	300	Fast

[a] Residues that are mutated in either [5-55]$_{Ala}$ or [30-51; 14-38]$_{V5A55}$ are denoted in parentheses.

[b] Observed to participate in an H bond in at least 1 of the crystal structures of native BPTI (Deisenhofer & Steigemann, 1975; Wlodawer et al., 1984, 1987.

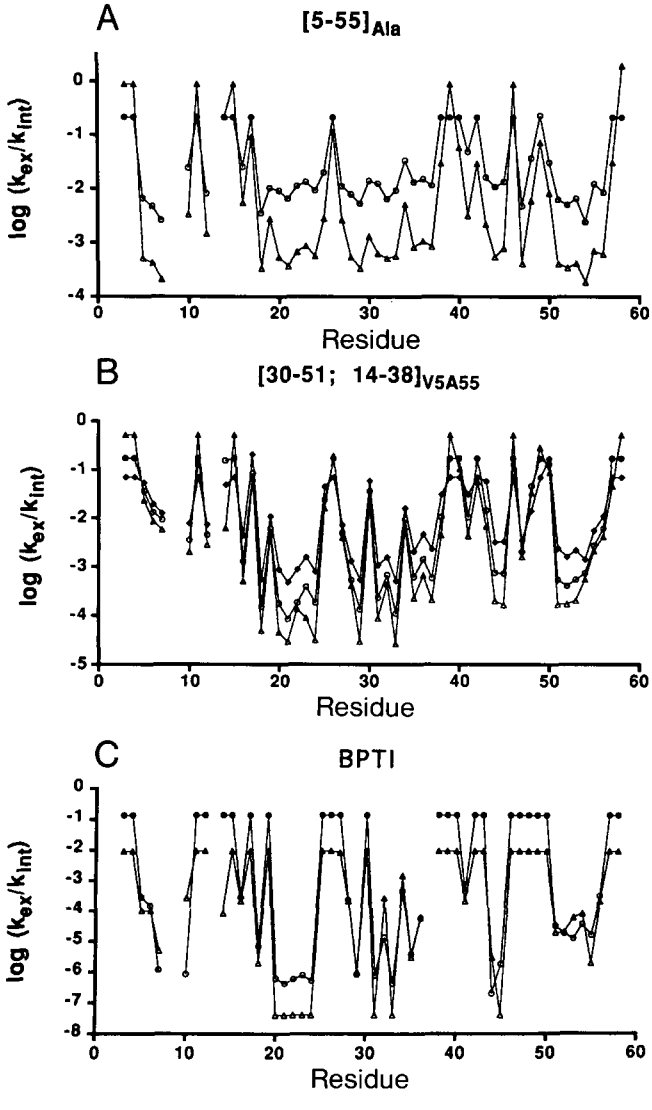

Fig. 4. Relative amide proton exchange rates plotted as log (k_{ex}/k_{int}) versus residue number at pH* 4.6 in (**A**) $[5\text{-}55]_{Ala}$ at 5 °C (triangles) and 20 °C (circles); (**B**) $[30\text{-}51; 14\text{-}38]_{V5A55}$ at 5 °C (triangles), 20 °C (circles), and 30 °C (diamonds); and at pH* 4.5 in (**C**) BPTI at 36 °C (circles) and 56 °C (triangles). The values for BPTI are taken from Wagner et al. (1984). Exchange rates that are too fast to measure are shown in filled symbols at the minimum rate of exchange for these residues.

Although almost all of the amide protons protected from exchange are the same in $[5\text{-}55]_{Ala}$ and $[30\text{-}51; 14\text{-}38]_{V5A55}$, some protons are protected to different extents in the 2 molecules. The most striking overall difference is that most of the protected amide hydrogens in $[5\text{-}55]_{Ala}$ are protected to a similar degree, whereas there is much greater variation in the protection factors for amide hydrogens in native BPTI (Wagner & Wüthrich, 1982a; Wagner et al., 1984) and $[30\text{-}51; 14\text{-}38]_{V5A55}$ (Fig. 4).

In both $[5\text{-}55]_{Ala}$ and $[30\text{-}51; 14\text{-}38]_{V5A55}$, all of the amide protons are more protected at 5 °C than at 20 °C. This difference is greater in $[5\text{-}55]_{Ala}$, which is slightly less stable than $[30\text{-}51; 14\text{-}38]_{V5A55}$; the T_m values are 42 °C and 60 °C, respectively (Staley & Kim, 1992; and data not shown). The extent of this increase in protection, however, is clearly different for different residues. In $[5\text{-}55]_{Ala}$, there is no obvious correlation between the temperature dependence of protection from amide proton exchange and structural features of the protein. In $[30\text{-}51; 14\text{-}38]_{V5A55}$, on the other hand, the amide hydrogens from the N-terminal 3_{10}-helix and from residues 54 and 55 in the C-terminal α-helix display a smaller change in protection factor with temperature than other residues involved in secondary structure. This difference is even more pronounced between 20 °C and 30 °C (Fig. 4).

Stable formation of native-like structures

In both $[5\text{-}55]_{Ala}$ and $[30\text{-}51; 14\text{-}38]_{V5A55}$, 25 of the 29 backbone amide hydrogens involved in H bonds in the crystal structures of native BPTI (Deisenhofer & Steigemann, 1975; Wlodawer et al., 1984, 1987) are protected from exchange by greater than 100-fold at 5 °C (Table 1). Of the remaining 4 H bonded residues, three (Phe 4, Lys 26, and Asp 50) are also not protected significantly in native BPTI (Wagner et al., 1984). The additional H bonded amide hydrogens that are not protected substantially from exchange are Val 5 in the case of $[30\text{-}51; 14\text{-}38]_{V5A55}$ and Ala 38 in $[5\text{-}55]_{Ala}$. In both cases, these residues are sites of mutation and the amide protons are protected slightly (over 30-fold at 5 °C).

Ten additional amide protons are highly protected in both $[5\text{-}55]_{Ala}$ and $[30\text{-}51; 14\text{-}38]_{V5A55}$. All of these protons are either considerably buried in the crystal structure of native BPTI (Deisenhofer & Steigemann, 1975; Wlodawer et al., 1984, 1987) or are protected in native BPTI despite exposure to solvent in the crystal structures (Wagner & Wüthrich, 1982a; Wagner et al., 1984; Tüchsen & Woodward, 1985). The amide proton of Ser 47 is significantly protected from exchange in both variants described here and is among the most protected amide protons in a partially folded variant of BPTI retaining only the 30-51 disulfide bond (Staley, 1993). Although Ser 47 has not been shown previously to be protected from exchange in native BPTI, it is buried in the crystal structure (Chothia & Janin, 1975; Wagner & Wüthrich, 1982a). Additionally, in $[5\text{-}55]_{Ala}$, Ala 30 is also protected more than 100-fold at 5 °C.

X-ray crystallographic studies have shown that $[30\text{-}51; 14\text{-}38]_{V5A55}$ has the same global fold as native BPTI (A.A. Kossiakoff, pers. comm.). Similarly, a previous ^{1}H NMR study showed that $[5\text{-}55]_{Ala}$ retains native structure (Staley & Kim, 1992). Our results show that stable, native-like folding occurs in both variants in solution.

Discussion

Comparison of amide proton exchange properties

One remarkable feature of amide proton exchange in $[5\text{-}55]_{Ala}$, as compared to native BPTI and $[30\text{-}51; 14\text{-}38]_{V5A55}$, is that all of the amide hydrogens involved in H bonds are protected to a similar extent, suggesting that exchange occurs predominantly via a route that involves global unfolding. It is particularly striking that at 5 °C, the N-terminal 3_{10}-helix and residues 54-56 of the C-terminal α-helix are more protected from exchange in $[5\text{-}55]_{Ala}$ than in $[30\text{-}51; 14\text{-}38]_{V5A55}$, even though $[5\text{-}55]_{Ala}$ is less stable than $[30\text{-}51; 14\text{-}38]_{V5A55}$. The differences in the extent of protection from exchange can be explained by differences in the subdomains that are likely to be populated in [30-51;

14-38]$_{V5A55}$ and [5-55]$_{Ala}$ (see also Clarke et al., 1993; Kim et al., 1993; Woodward, 1993).

BPTI contains a subdomain comprising the antiparallel β-sheet and C-terminal α-helix (Oas & Kim, 1988). Peptides corresponding to this subdomain, termed PαPβ, are folded in the presence of the 30-51 disulfide bond (Oas & Kim, 1988), and this subdomain is present in partially folded variants of BPTI containing only the 30-51 disulfide bond (Staley, 1993; van Mierlo et al., 1993; Staley & Kim, 1994). The N-terminal 3_{10}-helix is not part of the PαPβ subdomain. In [30-51; 14-38]$_{V5A55}$, the high degree of protection in part of the α-helix and the antiparallel β-sheet, taken together with the more local unfolding behavior of the 3_{10}-helix (Fig. 4B), suggests that the PαPβ region is a stable subdomain in [30-51; 14-38]$_{V5A55}$.

The 5-55 disulfide bond connects the N-terminal 3_{10}-helix to the C-terminal α-helix. Peptide models containing these secondary structures, together with the 5-55 disulfide bond, fail to fold without inclusion of a substantial portion of the central β-sheet of BPTI (Staley & Kim, 1990). Thus, although a peptide model, termed PαPγ, of a subdomain of BPTI containing the 5-55 disulfide can be made, at least part of each unit of BPTI secondary structure is required for folding (Staley & Kim, 1990). This requirement might explain the increased contribution of global unfolding to the exchange properties of [5-55]$_{Ala}$.

Another difference between the patterns of protection in [5-55]$_{Ala}$ and [30-51; 14-38]$_{V5A55}$ is the rate of exchange of residue 30 in the β-sheet. In native BPTI and [30-51; 14-38]$_{V5A55}$, Cys 30 is disulfide-bonded to Cys 51. In [5-55]$_{Ala}$, Cys 30 is mutated to alanine. The amide proton of Cys 30 exchanges rapidly in native BPTI (Wagner et al., 1984), [30-51; 14-38]$_{V5A55}$ (Figs. 3B, 4B), and a modified form of BPTI in which cysteines 14 and 38 have been blocked by iodoacetamide (Wagner et al., 1984). In [5-55]$_{Ala}$, however, the amide proton of Ala 30 is highly protected from exchange (Figs. 3A, 4A). This difference might arise from a change in dynamics near residue 30 in [5-55]$_{Ala}$, or from a conformational change upon removal of the 30-51 disulfide bond. Both solution and X-ray structures have been solved for a mutant BPTI in which both Cys 30 and Cys 51 have been mutated to alanines (Eigenbrot et al., 1990, 1992; Hurle et al., 1992). Although the mutation results in slight changes in conformation at the interface between the α-helix and β-sheet (Eigenbrot et al., 1990), there does not appear to be an obvious structural explanation for the dramatic increase in protection from amide proton exchange for Ala 30 in [5-55]$_{Ala}$.

Implications for the folding pathway of BPTI

A key feature of the oxidative folding pathway of BPTI (Creighton, 1977; Creighton & Goldenberg, 1984; Weissman & Kim, 1991, 1992b) is that an initially formed 2-disulfide intermediate, [30-51; 14-38], cannot oxidize directly to native BPTI, but instead rearranges to another 2-disulfide intermediate before forming a third disulfide bond (Creighton, 1977; Creighton & Goldenberg, 1984; Weissman & Kim, 1991). It has been proposed (Weissman & Kim, 1991, 1992a) that premature formation of native structure during the folding of BPTI buries the free thiols of Cys 5 and Cys 55, rendering them inaccessible to external oxidizing agents such as glutathione, thereby inhibiting formation of a third native disulfide bond in [30-51; 14-38] (see also Goto & Hamaguchi, 1981). The amide proton exchange properties of [30-51; 14-38]$_{V5A55}$ suggest that native structure, including that around residues 5 and 55, inhibits amide proton exchange much like it might inhibit disulfide chemistry.

Similarly, the 1-disulfide folding intermediate, [5-55], does not readily form the 30-51 disulfide bond. Again, this behavior can be explained (Staley & Kim, 1990, 1992; van Mierlo et al., 1991b) by native structure burying Cys 30 and Cys 51, inhibiting access of external oxidizing agents to these thiols. A previous NMR study of [5-55]$_{Ala}$ (Staley & Kim, 1992), and a related study of [5-55]$_{Ser}$ in which the cysteines other than 5 and 55 were replaced with serines (van Mierlo et al., 1991b), demonstrated that [5-55] folds into essentially the same conformation as native BPTI. The pattern of protection from amide proton exchange in [5-55]$_{Ala}$ (Fig. 4A) strengthens this conclusion.

In summary, our results demonstrate that variants of BPTI containing different disulfide bonds show local differences in the extent of protection from amide proton exchange, indicating that there are substantial local differences in the dynamics of the folded structure. Nonetheless, the high degree of protection of the same residues in [5-55]$_{Ala}$, [30-51; 14-38]$_{V5A55}$, and native BPTI underscores the conclusion that none of the disulfide bonds is absolutely required for BPTI to adopt its native fold in solution.

Materials and methods

Protein expression and purification

The gene encoding [30-51; 14-38]$_{V5A55}$ was produced by oligonucleotide-directed mutagenesis (Kunkel et al., 1987) of a gene encoding BPTI (D. Nguyen, J.P. Staley, & P.S. Kim, unpubl.), which was synthesized with convenient restriction sites and optimal codon usage for *Escherichia coli* and ligated into the *Nde* I/*Bam*H I site of pAED4, a pUC-based T7 expression plasmid with an F1 origin (Studier et al., 1990; Doering, 1992). The resulting plasmid is called pV5A55BPTI. The plasmid encoding [5-55]$_{Ala}$, denoted p5-55, is described elsewhere (M.-H. Yu, J.S. Weissman, & P.S. Kim, submitted). Unlabeled [5-55]$_{Ala}$ and [30-51; 14-38]$_{V5A55}$ were expressed essentially as described previously for [5-55]$_{Ala}$ (Staley & Kim, 1992) except that rifampicin was omitted. ^{15}N-labeled proteins were expressed in minimal M9 media, containing 1 g/L ^{15}N-ammonium sulfate (99.7% ^{15}N; Isotec, Ohio) and 0.5 mg/L thiamine (McIntosh & Dahlquist, 1990). Cells were induced at an OD_{600} of ~0.8 and harvested 3 h after induction. Both variants of BPTI form inclusion bodies upon expression in *E. coli*. Inclusion bodies containing [30-51; 14-38]$_{V5A55}$ were recovered and solubilized as for [5-55]$_{Ala}$ (Staley & Kim, 1992). Reduced [30-51; 14-38]$_{V5A55}$ was then purified by reversed-phase HPLC (Weissman & Kim, 1991) and the pure, reduced protein was refolded at a concentration of 10 μM in degassed folding buffer (100 mM Tris, pH 8.7, 1 mM EDTA, 5 mM reduced glutathione, 0.5 mM oxidized glutathione) containing 15% glycerol (Cleland, 1991) at room temperature and was subsequently repurified by reversed-phase HPLC. [5-55]$_{Ala}$ was purified and refolded as described previously (Staley & Kim, 1992). The identity of each variant was confirmed by laser desorption mass spectrometry on a Finnegan Lasermat and found to be within 2 Da of the calculated mass.

NMR spectroscopy and resonance assignments

Spectra were collected at protein concentrations of ~4 mM (pH 4.6) in the absence of buffer or salt. ^{1}H chemical shifts

were referenced to trimethylsilylpropionic acid (DeMarco, 1977), and the ^{15}N shifts referenced indirectly to NH_4^+ (Levy & Lichter, 1979). Data were collected on a Bruker AMX 500-MHz spectrometer. Water was presaturated for 1 s and 1,024 data points were collected in the $t2$ dimension; 150–256 increments were used in the $t1$ dimension. Data from 2D HSMQC-NOESY experiments were collected with a mixing time of 150 ms; data from 2D HSQC-TOCSY experiments were collected with mixing times of 80 and 110 ms (Gronenborn et al., 1989; McIntosh et al., 1990).

Strong similarity with spectra of BPTI (Wagner & Wüthrich, 1982b; Tüchsen & Woodward, 1987; Wagner et al., 1987; van Mierlo et al., 1991a) facilitated the assignment of resonances in spectra of $[30\text{-}51; 14\text{-}38]_{V5A55}$. At least 1 unambiguous sequential NOE (Wüthrich, 1986) was observed for each residue.

Equilibrium sedimentation

Measurements were made in Beckman 6-sector, 12-mm-pathlength epoxy cells in a Beckman XL-A 90 analytical ultracentrifuge. Samples were dialyzed against 20 mM acetic acid, pH 4.6. Data were collected at 5 °C at 3 wavelengths (249, 261, and 272 nm), rotor speeds (37, 41, and 45 krpm), and initial concentrations (50, 100, and 200 μM) as determined by tyrosine and cystine absorbance (Edelhoch, 1967). The final concentrations varied continuously between 25 μM and 400 μM. The program HID4000 (Johnson et al., 1981) was used to calculate molecular weights by simultaneously fitting 15 data sets to a single molecular weight, 15 intercepts, 14 offsets, and a single second virial coefficient. The residuals showed no systematic variation. Nonideality was observed as a decrease in the apparent molecular weight as a function of increasing protein concentration, requiring the use of the second virial coefficient as a fitting parameter. This nonideality most likely results from the large ratio of net charge to molecular weight (Williams et al., 1958) at the low ionic strength of the buffer used for NMR. The density of solutions and partial specific volumes of protein species were calculated using values from Laue et al. (1992). An analysis of $[5\text{-}55]_{Ala}$ yields a molecular weight of 6,246 Da ± 5% (95% confidence; calculated, 6,519 Da) and $[30\text{-}51; 14\text{-}38]_{V5A55}$ yields a molecular weight of 6,571 Da ± 4% (95% confidence; calculated 6,610 Da). A similar analysis of native BPTI yielded comparable results (Staley & Kim, 1994).

Amide proton exchange

Fully protonated samples of $[30\text{-}51; 14\text{-}38]_{V5A55}$ or $[5\text{-}55]_{Ala}$ were adjusted to pH 4.6 and lyophilized prior to initiating H-D exchange by dissolving the samples in exchange buffer (20 mM $NaCD_3COO$, pH* 4.6). (pH* refers here to meter readings in D_2O solutions using a glass pH electrode, without correction for isotope effects.) The temperature was maintained in circulating water baths between later time points. The protein concentration was ~3 mM.

Rates of H-D exchange were measured by recording ^{15}N-^1H HSQC spectra with 2–4 transients per increment, 2,048 real data points, an $\omega 2$ spectral width of 6,250 Hz, a total recycle delay of 1 s, and 128 increments of $t1$ (Norwood et al., 1990). ^{15}N decoupling during the acquisition was achieved with WALTZ-16 (Shaka et al., 1983). The residual HOD peak was suppressed with low power presaturation during the recycle delay.

Data analysis

Amide proton decays were followed by measuring peak volumes in ^{15}N-^1H HSQC spectra. The volumes were normalized to peak areas of the nonexchangeable resonances of the Y21 α or Y23 ϵ protons in 1D ^1H spectra collected immediately prior to each HSQC spectrum except the first six. One 1D spectrum was collected immediately following the first 6 experiments. The amide proton exchange rates, k_{ex}, were determined using the curve-fitting routine in Kaleidograph (Abelbeck software) by fitting the data to the 3-parameter fit, $I(t) = I(\infty) + I(0) \times \exp(-k_{ex} * t)$, where $I(t)$ is the intensity at time (t) after addition of deuterated solvent to the protein. Values for $I(\infty)$ were 60–200-fold lower than $I(0)$.

Predicted constants for the intrinsic rate of base-catalyzed exchange are based on data for H-D exchange in poly-D,L-alanine (Englander et al., 1979) with corrections for the effects of nearest neighbor side chains on intrinsic exchange rates (Molday et al., 1972; Robertson & Baldwin, 1991). The second-order rate constant for base-catalyzed exchange was modified to account for the isotope effect on the ionization of D_2O (Covington et al., 1966; Roder et al., 1985; Robertson & Baldwin, 1991). For calculation of intrinsic rates of exchange at temperatures other than 20 °C, the activation enthalpy was taken to be 2.8 kcal/mol. The pK_{D_2O} was calculated at different temperatures using the relationship (Covington et al., 1966) $pK_{D_2O} = 4{,}913(T)^{-1} - 7.60 + 0.02009(T)$.

Acknowledgments

We thank A.A. Kossiakoff for sharing with us the X-ray crystal structure coordinates of $[30\text{-}51; 14\text{-}38]_{V5A55}$ prior to publication; L.P. McIntosh, C.J. McKnight, and Z.-Y. Peng for help with NMR spectroscopy; and P.B. Harbury, K.J. Lumb, D.L. Minor, J.P. Staley, and J.S. Weissman for helpful discussions. B.A.S. was supported by the National Science Foundation predoctoral fellowship program and by a National Institutes of Health training grant (T32AI07348). This research was supported by a grant from the National Institutes of Health (GM41307).

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