Ultraviolet Difference Spectroscopy of Intermediates Trapped in Unfolding and Refolding of Bovine Pancreatic Trypsin Inhibitor†

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ABSTRACT: The solvent surface accessibilities of the aromatic amino acids of bovine pancreatic trypsin inhibitor have been examined after the protein has been trapped at various stages of unfolding and refolding. Two types of near-ultraviolet difference spectroscopy were used in making these measurements. One type compares the near-ultraviolet spectrum of each protein with the spectrum of the native inhibitor; the other is solvent perturbation spectroscopy. The two types of difference spectroscopy utilized are compared and found to be equivalent measures of tyrosine solvent exposure if the perturbation spectra are corrected for a probable contribution by buried residues. The experimental values for tyrosine solvent exposure in the native inhibitor are in agreement with those calculated from its crystal structure. The results of these studies identify an order in which the four tyrosines and the four phenylalanines of bovine pancreatic trypsin inhibitor are removed from solvent as refolding proceeds. The relative solvent accessibilities of the aromatic residues suggest an ordering in which the protein chain obtains compact globular structures.

There is virtually no information about how proteins fold to their final forms, although it has been recognized that proteins cannot sample all theoretically possible conformations during folding, but must fold through a finite number of conformations (Levinthal, 1968). Therefore, knowledge of the intermediate conformational states which occur during folding is necessary to understand how a protein's final conformation is obtained. There have been numerous studies of the processes of protein unfolding and refolding (reviewed by Baldwin, 1975; Pace, 1975; Anfinsen & Scheraga, 1975; Némethy & Scheraga, 1977; Creighton, 1978), but the only refolding pathway experimentally determined is that for bovine pancreatic trypsin inhibitor (BPTI)[1] which is based on the disulfide bonds broken and made during the unfolding–refolding process.

Native BPTI has three disulfide bonds, linking cysteines-30 and -51, -5 and -55, and -14 and -38 (Kassell & Laskowski, 1965; Anderer & Hörnle, 1966). The BPTI refolding pathway has been determined by trapping the intermediate states which accumulate kinetically, quenching the thiol–disulfide exchange process that occurs during folding, and carboxymethylating any remaining free sulfhydryls. These trapped intermediate states have been isolated, their disulfide bonds identified, and their positions and kinetic importance on the folding pathway defined (reviewed in Creighton, 1978).

The BPTI refolding pathway is illustrated in Figure 1; its important features are outlined here. Two one-disulfide-bond intermediates, (30–51) and (5–30), accumulate kinetically at early stages of refolding because they are thermodynamically most stable; (30–51), which has a native-like disulfide bond, is slightly more stable (0.6 kcal/mol) than is (5–30), which has a disulfide not found in native BPTI. Refolding to the two-disulfide stage occurs in (30–51) by formation of any of three second bonds involving cysteines-5, -14, and -38, yielding the species (30–51, 14–38), (30–51, 5–14), and (30–51, 5–38). Cysteine-55 is prevented from participating in disulfide bond formation at this stage of refolding.

Intermediate (30–51, 14–38) contains two of the three disulfides found in native BPTI, but cannot readily form the third disulfide, cystine-5–55. Instead (30–51, 14–38) is converted by disulfide rearrangement to either intermediate (30–51, 5–14) or intermediate (30–51, 5–38). These two intermediates, which can also be formed directly from the one-disulfide bond intermediates, undergo slow disulfide rearrangements to form the intermediate (30–51, 5–55). These two intramolecular rearrangements are the slowest steps of refolding and occur at approximately equal rates. Intermediate (30–51, 5–55) can readily complete refolding by rapid formation of the 14–38 disulfide.

In addition to (30–51, 5–55) being generated and trapped during refolding, a species containing the same disulfide bonds can be obtained by reduction of the 14–38 disulfide of native BPTI under nondenaturing conditions, followed by the carboxymethylation of cysteines-14 and -38 (Kress & Laskowski, 1967; Liu & Meienhofer, 1968; Creighton, 1975b, 1977a). This selective reduction is the first step in the unfolding process. This form of the inhibitor is designated N^{SCM}_{SCM} as it has been shown to be very similar to native BPTI in conformation (Vincent et al., 1971; Brunner et al., 1974; Snyder et al., 1976; Creighton et al., 1978; Creighton, 1978; Wagner et al., 1979a,b; Wagner & Wüthrich, 1979).

The disulfide bonds formed, and not formed, during refolding are a consequence of the conformations that exist during refolding. While the kinetics and relative stabilities of these intermediates are known (Creighton, 1977b), relatively little is known about the conformations of the intermediates.

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[1] Abbreviations used for proteins: BPTI, bovine pancreatic trypsin inhibitor, irrespective of the states of the cysteine residues. The inhibitor, prior to denaturation and reduction, is referred to as native BPTI. The inhibitor after reoxidation and renaturation is referred to as refolded BPTI. R is the fully denatured and reduced inhibitor with the six cysteines carboxyamidomethylated. N^{SCM}_{SCM} is native BPTI after selective reduction and carboxymethylation of cystine-14–38. Other forms of BPTI, i.e., the refolding intermediates, are designated by the residue numbers of the cysteine residues involved in disulfide bridges. Other abbreviations: *A*, absorbance; Pipes, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); Tris, tris(hydroxymethyl)aminomethane; UV, ultraviolet; NMR, nuclear magnetic resonance.

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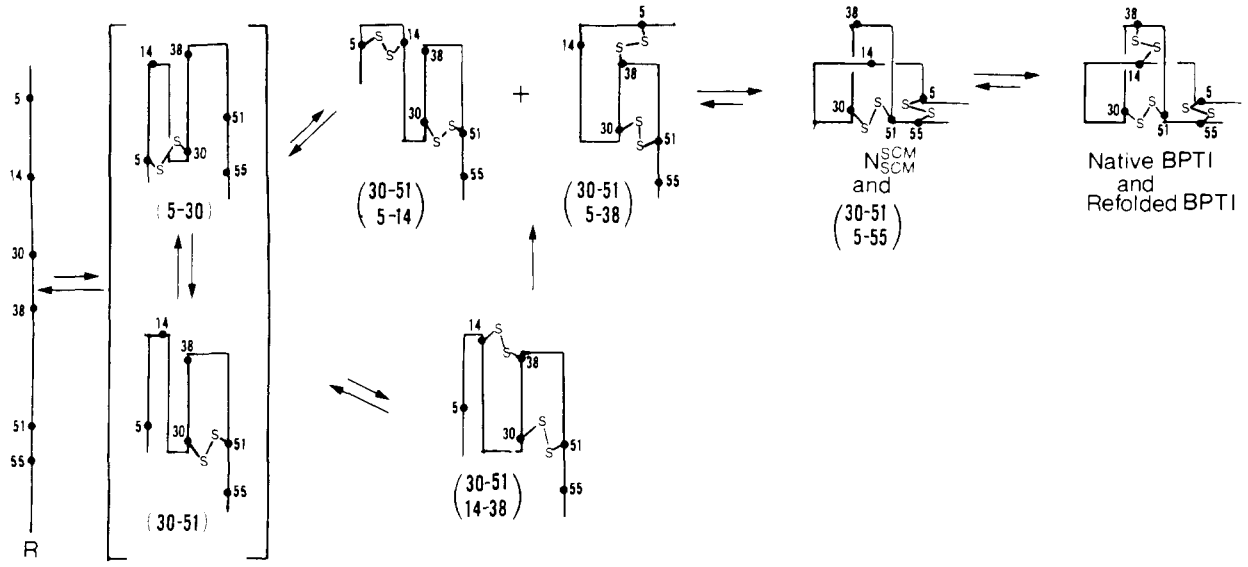

FIGURE 1: A schematic diagram of the BPTI pathway of folding and unfolding. The solid line at the left of the diagram represents the polypeptide backbone, with the positions of the six cysteine residues as indicated. The diagrammatic representations of the species containing the disulfides 30–51 and 5–55 and the disulfides 30–51, 5–55, and 14–38 approximate the folded conformation of native BPTI. The diagrammatic representations of the other conformers are arbitrary, except for the relative positions of the disulfide bonds. The cystine pairs are shown below each diagram of the intermediates. The brackets around the one-disulfide-bond intermediates indicate that they are in rapid equilibrium. The "+" between (30–51, 5–14) and (30–51, 5–38) signifies that both are formed directly from the one-disulfide-bond intermediates, that both are converted directly to (30–51, 5–55), that either or both are intermediates in the rearrangement of (30–51, 14–38) to (30–51, 5–55), and that they elute as a mixture during carboxymethylcellulose chromatography. (Taken in modified form from Creighton, 1977a.)

To date, only the propensities of the intermediates to bind to antibodies raised independently against native BPTI and the fully reduced and carboxymethylated BPTI have been reported (Creighton et al., 1978).

We report here conformational studies on BPTI and its refolding intermediates using the technique of near-UV difference spectroscopy; circular dichroism measurements will be described later. Spectral techniques rapidly provide conformational information concerning secondary structure and the relative environments of aromatic amino acids and cystines. Such studies are particularly meaningful with BPTI, as it has been the subject of extensive crystallographic, physical, and theoretical studies (e.g., Huber et al., 1970, 1971; Vincent et al., 1971; Karplus et al., 1973; Brunner et al., 1974; Deisenhofer & Steigemann, 1974, 1975; Gelin & Karplus, 1975, 1979; Snyder et al., 1976; McCammon et al., 1979; Woody, 1978; Wüthrich & Wagner, 1978; Wagner & Wüthrich, 1979). Here we document the solvated states of the four tyrosines of BPTI, tyrosines-10, -21, -23, and -35, and the four phenylalanines, phenylalanines-4, -22, -33, and -45, in each of the refolded states of BPTI using two types of near-UV difference spectroscopy. One of the techniques, referred to as comparison spectroscopy, compares the near-UV spectrum of each refolding state with the spectrum of native BPTI. The other method is solvent perturbation spectroscopy, with ethylene glycol as the perturbant (Herskovits & Laskowski, 1962).

Materials and Methods

Materials. Ethylene glycol was obtained from J. T. Baker. Piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (Pipes) was a product of Sigma Chemical Co. Glycyl-L-phenylalanylglycine (Gly-Phe-Gly) and glycyl-L-tyrosylglycine (Gly-Tyr-Gly) were obtained from Vega-Fox Biochemicals. Both tripeptides were found to be pure by the criteria of thin-layer chromatography and amino acid analysis.

Proteins. Native BPTI (Trasylol) was a gift of Bayer AG. Two lots of the inhibitor were used, GOS 746/31 and SMU 681. The first lot was used without further purification. The second lot, SMU 681, contained an unidentified impurity which absorbed in the near-UV. This impurity was removed by chromatography of the protein in H_2O on Chelex 100 (Bio-Rad Laboratories). The purified BPTI solution was then titrated to neutrality with HCl and lyophilized.

N^{SCM}_{SCM}, with cysteines-14 and -38 blocked by carboxymethylation, was prepared and purified as described elsewhere (Creighton, 1975b, 1977a).

R, with the six thiol groups carboxyamidomethylated, was prepared by reducing native BPTI in 6.0 M guanidine hydrochloride, 0.2 M Tris-HCl, pH 8.7, and 30 mM dithiothreitol for 3 h at room temperature, followed by the addition of a 2-fold excess of iodoacetamide. After incubation in the dark for 3 h, R was isolated by gel filtration in 0.01 M HCl.

Intermediates with one or two disulfide bonds were trapped by alkylation with iodoacetic acid during the refolding process, using glutathione as the disulfide reagent. The intermediates were then isolated by chromatography on carboxymethylcellulose (Creighton, 1975a). The proteins were desalted by gel filtration and isolated by lyophilization. All of the intermediates are isolated as homogeneous species except for (30–51, 5–14) and (30–51, 5–38), which are not separated by ion-exchange chromatography; these two intermediates have been examined as a mixture.

The lyophilized proteins were dissolved in 6.0 mM Pipes, pH 6.8, and filtered using a Millipore filter, No. HATF 01300, before the spectra were recorded.

Comparison Spectroscopy. Comparison spectra were recorded on a Cary 14 spectrophotometer using 1.0-cm cuvettes thermostated at 25 °C and (usually) the 0.1-*A* scale expansion. The automatic slit width was employed. The dynode voltage was set at 2 and the slit control at 20. Native BPTI is the reference protein. The sample solution and the native BPTI solution for a given comparison spectrum were diluted so that the $A_{\lambda_{max}}$'s of their respective near-UV absorption spectra were of equal intensity. This equality has been shown to be a good approximation for equivalent concentrations (Kosen, 1978). The spectra were recorded between 240 and 350 nm, but only the wavelength regions above 270 nm are reported here since

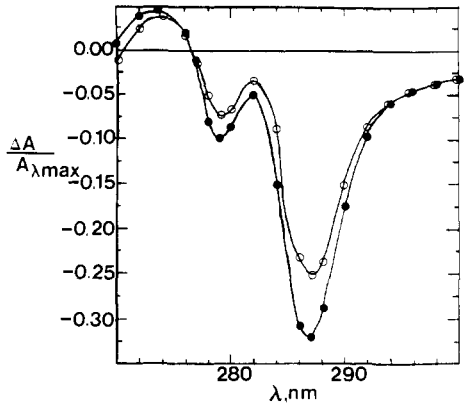

FIGURE 2: The near-UV comparison spectra of Gly-Tyr-Gly plus Gly-Phe-Gly vs. native BPTI (●) and R vs. native BPTI (○) in 6.0 mM Pipes, pH 6.8, at 25 °C. The concentrations of Gly-Tyr-Gly and Gly-Phe-Gly are four times the concentration of native BPTI so that the absolute concentrations of the model aromatic amino acids and the amino acids of native BPTI are identical.

the region below 270 nm is dominated by differential absorption of cystines, which obscures the differential phenylalanine absorption. Solutions of 6.0 mM Pipes, pH 6.8, were used as the base-line blanks. All spectra are the average of at least two samples. Spectra were standardized by dividing the differential absorbance, ΔA, by $A_{\lambda_{max}}$ of the absorption spectrum at 2.5-nm intervals and at all maxima and minima.

The comparison spectra for (30–51, 5–55) and refolded BPTI were not determined in the manner outlined above but were calculated directly from their and native BPTI's absorption spectra. This procedure was necessary because the absorption spectra of (30–51, 5–55) and refolded BPTI contained an absorption tail of unknown origin between 320 and 350 nm which was not removed by ultracentrifugation or Millipore filtration. The absorbance at 320 nm for all samples of refolded BPTI and (30–51, 5–55) was always less than 5% of the absorption maximum in the near-UV region. Linear extrapolation of the tail into the absorbing region was routinely used to correct the absorption spectra. This correction gave consistent extinction coefficient values for the spectra of both refolded BPTI and (30–51, 5–55) at various concentrations. However, the presence of the tail between 320 and 350 nm prevented the direct measurement of their comparison spectra.

Solvent Perturbation Spectroscopy. Perturbation spectra were recorded essentially as outlined by Herskovits & Laskowski (1962). A Cary 118c spectrophotometer was used because the scale could be varied up to 0.02A at full expansion. Square 1.0-cm split cuvettes, thermostated at 25 °C, were used. The slit width was held constant at 0.1 mm. Protein solutions containing 0% and 20% ethylene glycol were prepared by 1:1 dilution with stock protein solutions and 6.0 mM Pipes, pH 6.8, without and with 40% ethylene glycol. Buffer blanks containing 0% and 20% ethylene glycol were prepared in the same manner. Each spectrum was recorded twice during a time period of approximately 40 min. Only those runs with two identical raw traces were used. The spectra of at least two different samples of each protein were recorded. The pH of each solution was measured after recording the spectrum; the values between solutions did not differ more than 0.1 pH unit. The spectra were standardized by dividing $^1/_2A_{\lambda_{max}}$ of the stock protein solution into ΔA at 2.5-nm intervals and at all maxima and minima.

There were no indications of conformational alterations due to the presence of ethylene glycol, as shown by comparison of the circular dichroism spectrum of each BPTI species in the absence and presence of the perturbant (Kosen, 1978).

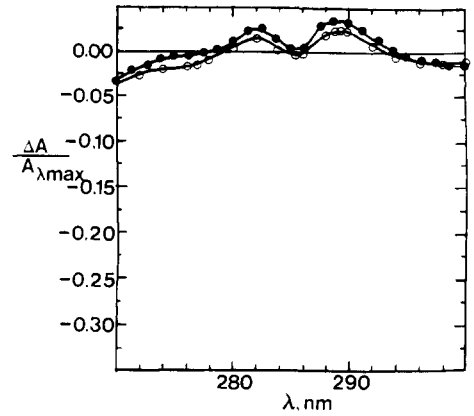

FIGURE 3: The near-UV comparison spectra of N_{SCM}^{SCM} vs. native BPTI (○) and (30–51, 5–55) vs. native BPTI (●) in 6.0 mM Pipes, pH 6.8, at 25 °C. The comparison spectrum, (30–51, 5–55) vs. native BPTI, was calculated from the respective absorption spectra as described under Materials and Methods.

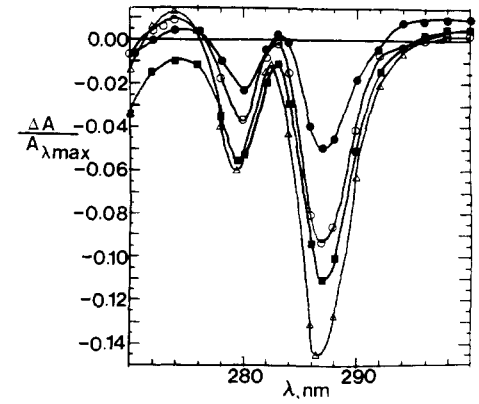

FIGURE 4: The near-UV comparison spectra of (5–30) vs. native BPTI (△), (30–51) vs. native BPTI (■), (30–51, 5–14) plus (30–51, 5–38) vs. native BPTI (○), and (30–51, 14–38) vs. native BPTI (●) in 6.0 mM Pipes, pH 6.8, at 25 °C.

Calculation of the Tyrosine Static Accessible Surface of Crystalline BPTI. The theoretical static accessible surface area of the BPTI aromatic amino acid residues (to water) was determined by a computer calculation first developed by Lee & Richards (1971) and employed by Chothia (1976) for BPTI. The results of these calculations were communicated to us by Dr. C. Chothia.

Results

Comparison Spectroscopy. The comparison spectra of R and Gly-Tyr-Gly plus Gly-Phe-Gly are shown in Figure 2. The comparison spectra of (30–51, 5–55) and N_{SCM}^{SCM} are shown together in Figure 3. All other comparison spectra are shown in Figure 4. The comparison spectrum for refolded BPTI was found to be insignificant at all wavelengths.

As expected, there are two differential absorption minima [or, in the spectra of (30–51, 5–55) and N_{SCM}^{SCM}, two maxima] in the tyrosine region (~270–285 nm) for each comparison spectrum. The value for $\Delta A/A_{\lambda_{max}}$ of each long-wavelength peak in each spectrum is given in Table I. The negative values of $\Delta A/A_{\lambda_{max}}$ indicate that the tyrosines of all BPTI refolding conformers, with the apparent exceptions of N_{SCM}^{SCM} and (30–51, 5–55), are more exposed to solvent than are the tyrosines of native BPTI. The positive values of the spectra of N_{SCM}^{SCM} and (30–51, 5–55) may indicate that the tyrosine of these two conformers are less exposed to solvent than those of native BPTI.

An attempt to quantitate the fractional exposures of the tyrosines was made by using either R or Gly-Tyr-Gly plus

Table I: Comparison Spectra Parameters

sample	$\Delta A/A_{\lambda_{max}}$ [a]	fractional exposure of the tyrosines[b] compared to R	fractional exposure of the tyrosines[b] compared to Gly-Tyr-Gly plus Gly-Phe-Gly
R	−0.252 ± 0.002	100	84
Gly-Tyr-Gly plus Gly-Phe-Gly	−0.321 ± 0.006		100
(5–30)	−0.146 ± 0.004	73	59
(30–51)	−0.112 ± 0.005	64	51
(30–51, 5–14) plus (30–51, 5–38)	−0.0933 ± 0.0104	60	47
(30–51, 14–38)	−0.0496 ± 0.0016	49	37
N^{SCM}_{SCM}	+0.0263 ± 0.003	29	19
(30–51, 5–55)	+0.035	27	16
refolded BPTI or native BPTI		36	25

[a] The error estimate is the average deviation. There is no error estimate made for the (30–51, 5–55) vs. native BPTI spectrum. [b] The fractional exposures are given as percentages. The fractional exposure values for refolded BPTI and native BPTI are calculated from the spectra of R and the model compounds as described in the text.

Gly-Phe-Gly as the standard for complete tyrosine solvent exposure. However, native BPTI could not be used as a standard for 100% tyrosine burial since it is known that the tyrosines of crystalline BPTI are partially exposed to solvent (Huber et al., 1970, 1971; Deisenhofer & Steigemann, 1974, 1975). Therefore, as suggested by Donovan (1969), an appropriate reference for the transition, 100% tyrosine burial → 100% tyrosine exposure, was taken as six times the absolute value of the long-wavelength maximum of the solvent perturbation spectrum of R or Gly-Tyr-Gly. (These two perturbation spectra are shown and discussed later.) With the assumption that the perturbation spectrum of either R or Gly-Tyr-Gly is an appropriate standard for 100% tyrosine solvent exposure, the absolute value of $\Delta A/A_{\lambda_{max}}$, for the hypothetical case of 100% tyrosine burial in a comparison spectra, would be 0.393 when R is used as the standard and 0.426 when Gly-Tyr-Gly is used as the standard. The fractional exposure of the tyrosines of native BPTI was then calculated by dividing the absolute value of $\Delta A/A_{\lambda_{max}}$ of the long-wavelength peak of R's or the Gly-Tyr-Gly plus Gly-Phe-Gly comparison spectrum by 0.393 or 0.426, respectively. These calculations indicate that the tyrosines of native BPTI are 36% exposed to solvent in comparison to the tyrosines of R and 25% exposed to solvent in comparison to the tyrosine of Gly-Tyr-Gly.

The fractional exposures of each of the other BPTI intermediates were calculated by a similar division followed by addition of either 36% or 25% depending on the choice of reference for 100% exposure. These tyrosine fractional exposures are given in the last two columns of Table I.

The comparison spectra indicate that the tyrosines become less exposed to solvent in the order R > (5–30) > (30–51) ≈ (30–51, 5–14) plus (30–51, 5–38) > (30–51, 14–38) > native BPTI = refolded BPTI ≳ N^{SCM}_{SCM} = (30–51, 5–55).

Solvent Perturbation Spectroscopy. The perturbation spectra of R, Gly-Tyr-Gly, and Gly-Phe-Gly are shown together in Figure 5. Both the tyrosine and the phenylalanine peaks in the spectra of R and the model peptides nearly overlap.

The perturbation spectra of native BPTI and refolded BPTI, shown in Figure 6, are virtually identical. The spectra of N^{SCM}_{SCM}

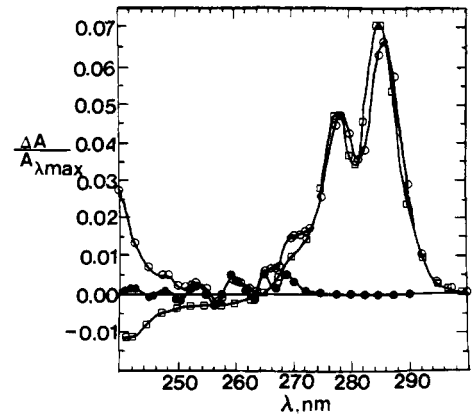

FIGURE 5: The solvent perturbation spectra of Gly-Tyr-Gly (□), Gly-Phe-Gly (●), and R (○) produced by 20% ethylene glycol in 6.0 mM Pipes, pH 6.8, at 25 °C. The spectrum of Gly-Phe-Gly is reduced by a factor of 195/1400 so that the relative magnitudes of the model compound spectra are proportional to the relative magnitudes that occur in the solvent perturbation spectra of the BPTI conformers.

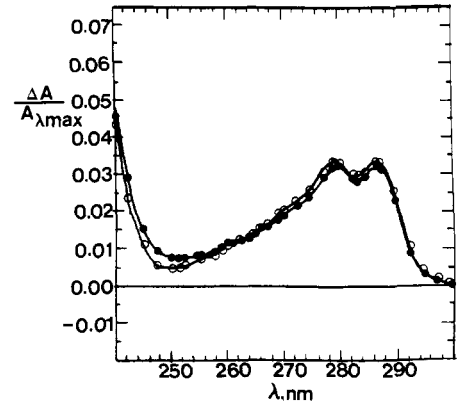

FIGURE 6: The solvent perturbation spectra of native BPTI (●) and refolded BPTI (○) produced by 20% ethylene glycol in 6.0 mM Pipes, pH 6.8, at 25 °C.

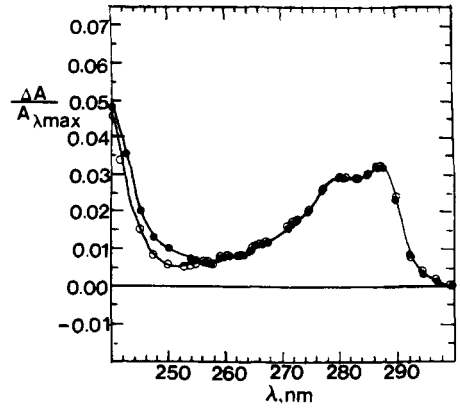

FIGURE 7: The solvent perturbation spectra of N^{SCM}_{SCM} (○) and (30–51, 5–55) (●) produced by 20% ethylene glycol in 6.0 mM Pipes, pH 6.8, at 25 °C.

and (30–51, 5–55), shown in Figure 7, are also identical and very similar to the spectra of native BPTI and refolded BPTI. The phenylalanine peaks of these four spectra are much less apparent than in the spectrum of R. The similar spectra of intermediate (30–51) and the mixture of intermediates (30–51, 5–14) plus (30–51, 5–38) are shown in Figure 8. Finally the spectra of (5–30) and (30–51, 14–38) are shown in Figure 9.

Qualitatively there appears to be a decrease in phenylalanine exposure in the order R > (5–30) > (30–51) ≈ (30–51, 5–14) plus (30–51, 5–38) ≈ (30–51, 14–38) > (30–51, 5–55) =

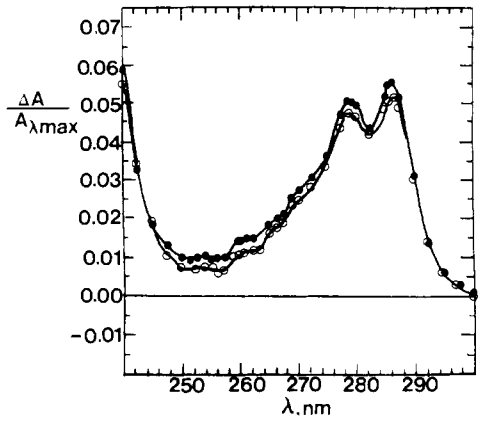

FIGURE 8: The solvent perturbation spectra of (30–51) (●) and (30–51, 5–14) plus (30–51, 5–38) (○) produced by 20% ethylene glycol in 6.0 mM Pipes, pH 6.8, at 25 °C.

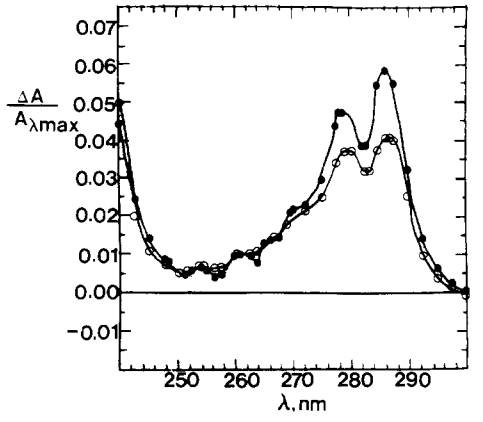

FIGURE 9: The solvent perturbation spectra of (5–30) (●) and (30–51, 14–38) (○) produced by 20% ethylene glycol in 6.0 mM Pipes, pH 6.8, at 25 °C.

$N^{SCM}_{SCM} \approx$ refolded BPTI = native BPTI. The fractional exposure of the tyrosines decreases approximately in the same order. However, the tyrosines of (30–51, 14–38) are significantly less exposed than those of (30–51, 5–14) plus (30–51, 5–38), whereas the phenylalanines of these three two-disulfide intermediates appear to have a similar degree of solvent exposure.

A fractional solvent exposure was calculated from the solvent perturbation spectra by division of $\Delta A/A_{\lambda_{max}}$ of the long-wavelength peak of each protein spectrum by $\Delta A/A_{\lambda_{max}}$ of the long-wavelength peak of the reference standard, either R or Gly-Tyr-Gly. The results of these calculations are given in the last two columns of Table II. The first column of that table gives the $\Delta A/A_{\lambda_{max}}$ values of the long-wavelength maximum for each BPTI conformer.

At least qualitatively, the removal of the phenylalanines and the tyrosines from solvent appears to occur in parallel. There is also qualitative agreement between the relative fractional tyrosine exposure for each BPTI conformer as determined by the two types of difference spectroscopy except in the cases of (30–51, 5–55) and N^{SCM}_{SCM}. Comparison spectroscopy indicates that the tyrosine(s) of these two-disulfide-bond intermediates are slightly less exposed to solvent than are those of native BPTI, whereas perturbation spectroscopy would, if only the first tyrosine maxima were examined, indicate no difference in the tyrosine exposure of native BPTI, refolded BPTI, N^{SCM}_{SCM}, and (30–51, 5–55). A possible rationale for this apparent disagreement is given below. A suggestion for correcting the quantitative disagreements between the comparison spectra and the perturbation spectra is also made under Discussion.

Table II: Solvent Perturbation Spectra Parameters

sample	$\Delta A/A_{\lambda_{max}}$ [a]	fractional exposure of the tyrosines[b] compared to R	fractional exposure of the tyrosines[b] compared to Gly-Tyr-Gly
R	0.0655 ± 0.0015	100	92
Gly-Tyr-Gly	0.071 ± 0.0011		100
(5–30)	0.0587 ± 0.0017	90	83
(30–51)	0.0554 ± 0.0004	85	78
(30–51, 5–14) plus (30–51, 5–38)	0.0521 ± 0.001	80	73
(30–51, 14–38)	0.0411 ± 0.0011	63	58
N^{SCM}_{SCM}	0.0331 ± 0.0014	51	47
(30–51, 5–55)	0.0329 ± 0.0003	50	46
refolded BPTI	0.0337 ± 0.0003	51	47
native BPTI	0.0322 ± 0.0006	49	45

[a] The error estimates for the spectra of refolded BPTI, (30–51, 5–55), (30–51), and (30–51, 14–38) are the average deviations. All other errors are calculated as the standard error of the mean. [b] The fractional exposures are given as percentages.

Table III: Theoretical Accessible Surface Area (A²) of the Tyrosines of Crystalline BPTI

atom[a]	Tyr-10	Tyr-21	Tyr-23	Tyr-35
N	1.4	0	0	0
C_α	0.9	0	0	0
C_β	2.3	0	0	0
C_γ	0	0	0	0
C_{δ_1}	15.2	0	0	0
C_{ϵ_1}	25.7	9	0	0
C_ζ	2.7	0.5	0	0
OH	25.1	36.3	7.1	6.7
C_{ϵ_2}	1.1	7.4	0	6.3
C_{δ_2}	0	0	0	0
C	0	0	0	0
O	0.7	0	1.8	0
total	75.1	53.2	8.9	13.0

[a] C_α, N, C, and O are the peptide backbone atoms.

Static Accessible Surface Area of the Aromatic Amino Acid Residues of Native BPTI. The accessible surface area of each tyrosine residue, and of the individual atoms, in the crystal structure of native BPTI is given in Table III. When the tripeptide Gly-Tyr-Gly is used as a model for 100% solvent exposure (Chothia, 1976), the total fractional solvent exposure of the tyrosine residues is calculated as 16%. If only the phenol ring of each tyrosine is considered, the fractional exposure is 31%. (The model for complete exposure in this case is the accessible surface area of Gly-Tyr-Gly minus the accessible surface area of Gly-Ala-Gly. The accessible surface area calculations for the two tripeptides were taken from the work of Chothia (1976).) Similar calculations indicate an average solvent accessibility for the phenylalanine residues as 11.8%, or as 15.6% if only the aromatic rings are considered.

A model of native BPTI is shown in Figure 10. The positions of the tyrosines and the phenylalanines are indicated. Two of the three disulfides can also be seen in this view of the protein model. Cystine-30–51 is partially obscured from view at this angle.

Discussion

Relevance to the Pathway of Unfolding and Refolding. The results of these spectral studies show that the aromatic amino acids of the trapped refolding intermediates of BPTI are increasingly shielded from solvent in the order R > (5–30) > (30–51) ≈ (30–51, 5–14) plus (30–51, 5–38) > (30–51, 14–38) > (30–51, 5–55) = $N^{SCM}_{SCM} \approx$ refolded BPTI = native BPTI.

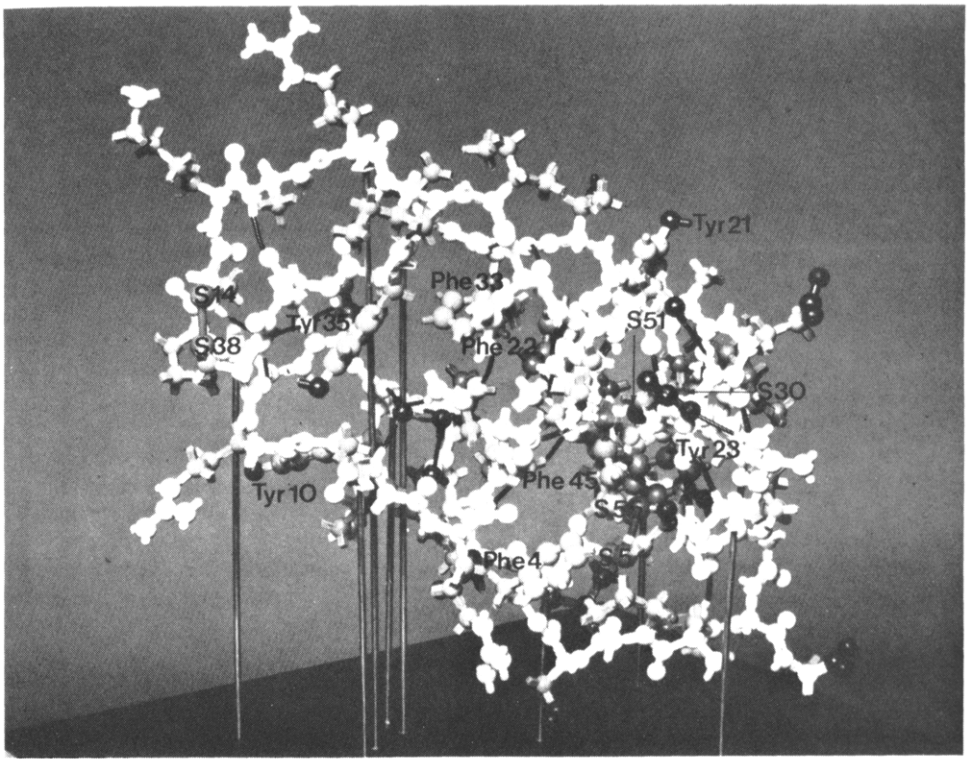

FIGURE 10: The native conformation of BPTI with the tyrosines, phenylalanines, and cystines identified.

This order holds in general for both the tyrosines and the phenylalanines. Both types of spectroscopy indicate that the solvated states of the aromatic residues of native and refolded BPTI are the same. Similarly, the solvated states of the aromatic residues of (30–51, 5–55) and N^{SCM}_{SCM} are also identical. The identical solvent exposure for each of these pairs is not surprising since both species in each pair of proteins have the same disulfide bonds, hold the same position on the unfolding–refolding pathway, and have been shown to have certain other physical properties in common (Creighton, 1978). However, circular dichroism measurements (Kosen, 1978) indicate that each of the proteins in the above pairs do not have their tyrosine residues in identical environments. Circular dichroism is a more sensitive measure of tyrosine environments, and since the differences found in the circular dichroism measurements are small (5% change in intensity), it is not surprising that difference spectroscopy has discerned no differences in the solvated states of native and refolded BPTI nor of N^{SCM}_{SCM} and (30–51, 5–55).

That the relative solvent exposures of the aromatic residues of native BPTI, refolded BPTI, N^{SCM}_{SCM}, and (30–51, 5–55) are similar was expected as reduction and carboxymethylation of the 14–38 disulfide had been shown to have no effect on the aromatic Raman bands of native BPTI (Brunner et al., 1974) nor on the pK of any of the tyrosines after the two cysteines are carboxyamidomethylated (Snyder et al., 1976). Further, reduction and carboxyamidomethylation of cystine-14–38 affected only the NMR chemical shifts of tyrosines-10 and -35 in a manner that indicated a possible increased solvent exposure of only tyrosine-10 (Snyder et al., 1976; Wagner et al., 1979a).

R has its aromatic residues in environments that are more exposed to solvent than those of any other BPTI conformer. The comparison spectra and the solvent perturbation spectra of R and the model compounds Gly-Phe-Gly and Gly-Tyr-Gly are not identical, however. The differences in the spectra of R and the spectra of the model compounds could be due either to specific partial burial of one or more of the tyrosines (up to 64% of one tyrosine buried in R) or, more likely, to the bulkiness of certain neighboring side groups. For example, tyrosines-21 and -23 are positioned in the primary sequence as Arg-20–Tyr-21–Phe-22–Tyr-23, a linear sequence of four large side chains. BPTI denatured in the presence of 8.0 M urea and β-mercaptoethanol did not show tyrosine NMR resonances identical with those of a model tyrosine compound (Karplus et al., 1973). Thus NMR spectroscopy also suggests some remaining hindrance in the free rotation and thus complete solvation of the tyrosines.

When the individual steps of refolding are compared, a greater decrease in aromatic residue solvent exposure occurs when (30–51), rather than (5–30), is formed from R, and when (30–51, 14–38) is formed from the one-disulfide intermediates. The refolding steps (30–51) → (30–51, 5–14) and (30–51) → (30–51, 5–38) showed only a small change in the solvated states of the aromatic amino acids, which indicates that formation of either the 5–14 or 5–38 disulfide does not significantly change the relative tyrosine environments of (30–51). Intermediate (30–51) has somewhat lower energy than (5–30) during folding, which would suggest more favorable conformational interactions, and is known from its electrophoretic mobility to be more compact (Creighton, 1978). Likewise, (30–51, 14–38) is more compact than (30–51, 5–14) and (30–51, 5–38), although their similar levels of accumulation during refolding indicate similar energies.

Again the general order of decreasing exposure to the solvent of the aromatic residues of the trapped intermediates in the refolding of BPTI is R > (5–30) > (30–51) $\approx$ (30–51, 5–14) plus (30–51, 5–38) > (30–51, 14–38) > (30–51, 5–55) = N^{SCM}_{SCM} $\approx$ refolded BPTI = native BPTI. The decrease in solvent accessibility for the aromatic residues suggests that the total polypeptide chain becomes more compact and globular (or less like the conformations of R) in the same order, since other portions of the polypeptide chain must be involved in the exclusion of the aromatic residues from the solvent. The

Table IV: Comparison of the Fractional Tyrosine Solvent Exposure Calculated from the Comparison Spectra and the Corrected Solvent Perturbation Spectra

sample	ratio[a]	fractional exposure of the tyrosines			
		compared to R		compared to Gly-Tyr-Gly	
		comparison spectra	perturbation spectra	comparison spectra	perturbation spectra
R	1.39	100	100	84	86
(5–30)	1.23	73	80	59	69
(30–51)	1.09	64	67	51	57
(30–51, 5–14) plus (30–51, 5–38)	1.09	60	63	47	53
(30–51, 14–38)	1.09	49	49	37	42
N^{SCM}_{SCM}	1.11	29	41	19	35
(30–51, 5–55)	1.13	27	41	16	35
refolded BPTI	1.0	36	37	25	32
native BPTI	1.0	36	35	25	30

[a] Ratio of the long- to the short-wavelength differential absorption peaks. The ratio for the Gly-Tyr-Gly perturbation spectrum is 1.49.

order of decrease in the solvent accessibilities of the aromatic residues is very similar to the general order of compactness observed electrophoretically and measured immunochemically by affinity to antibodies against native BPTI (Creighton et al., 1978). This affinity is a measure of the relative tendencies of the intermediates to adopt native-like conformations. However, the relative degree of solvent exposure is not the same as the order observed immunochemically for affinity to antibodies raised against R, nor is it the same as the order in refolding, where (30–51, 5–14) and (30–51, 5–38) have been concluded to be intermediate in the interconversion of (30–51, 14–38) to (30–51, 5–55). The order of affinity to antibodies raised against the fully reduced inhibitor is more nearly the same as the order of disulfide bond formation in the refolding pathway (Creighton et al., 1978). This difference between the order determined in the spectral studies and the immunochemical studies using antibodies against native BPTI vs. the immunochemical studies using antibodies against R and the order of disulfide bond formation during refolding suggests that the apparent compactness of the polypeptide chain is not the predominant factor in determining the order of refolding. Instead, it has been suggested that the pathway of refolding to the native-like state, i.e., formation of (30–51, 5–55), is determined primarily by the rate-determining energy barrier which separates the two-disulfide species (30–51, 5–14), (30–51, 5–38), and (30–51, 14–38) from the native-like state (Creighton et al., 1978).

Comparison of the Two Types of Difference Spectroscopy. Perturbation spectroscopy and to a lesser extent the type of measurement we have called comparison spectrscopy are acceptable measures of solvent accessibilities for the aromatic residues of proteins. These measurements normally are taken at face value as reliable estimates of solvent accessibility in a manner similar to the estimates made for the secondary structures of proteins based on far-UV circular dichroism spectroscopy. The pitfalls of using circular dichroism spectra to estimate secondary structures of proteins have become fairly well-known (e.g., Fasman 1973; Rosenkranz, 1974). However, the pitfalls of using difference spectroscopy to measure the solvent accessibilities of aromatic amino acids appear to have been less well recognized, especially relating to the contributions of buried residues to perturbation spectroscopy. Laskowski (1966) has emphasized that aromatic residues which are at distances of 5–10 Å beneath a protein's surface may potentially contribute to a solvent perturbation spectrum. More specifically, Brandts & Kaplan (1973) have experimentally shown that three tyrosines of ribonuclease which are chemically unreactive and therefore "inaccessible" to solvent contribute at least one-third of the differential absorbance to the long-wavelength peak in a 25% ethylene glycol perturbation spectrum of ribonuclease. The above examples appear to be the primary warnings that ethylene glycol solvent perturbation spectroscopy potentially overestimates the solvent accessibilities of aromatic residues.

The preceding section of this discussion, which dealt with the relative solvent accessibilities of the tyrosines (and phenylalanines) in relation to the positions of the intermediates on the refolding pathway, ignored the absolute numerical values of tyrosine solvent exposures derived from the perturbation and comparison spectra. Both types of spectroscopy indicated the same qualitative ordering of tyrosine solvent exposure for each BPTI refolding intermediate with the exceptions of N^{SCM}_{SCM} and (30–51, 5–55). However, for each intermediate, the absolute solvent accessibility, determined by the two types of spectroscopy, differed by about 20% on average. In all cases, the perturbation spectrum indicated the greater degree of solvent exposure. (The 20% difference in the values appears to be independent of the model chosen for 100% solvent exposure.)

We found the discrepancies between the two types of difference spectroscopy disturbing. Closer examination of our spectra (and the ribonuclease spectrum) indicated to us that the relatively inaccessible tyrosines that might not be directly in contact with solvent were increasingly contributing to the individual solvent perturbation spectra as refolding progressed. The reasoning for this conclusion derived from an examination of Brandts and Kaplan's work and is as follows: Examination of the ribonuclease spectrum shows a long to short tyrosine wavelength peak ratio of 1.29, whereas the ratio of the two peaks in the spectrum of *N*-acetyl-L-tyrosine ethyl ester, the model chosen in that case for 100% solvent exposure, is 1.76 (our calculations). Furthermore, the ratio measured for the spectrum of the three accessible tyrosines in ribonuclease as determined by Brandts and Kaplan is 1.6, a value which approximates the ratio found for the model compound spectrum. Comparison of the ratio of the long- to short-wavelength peaks of the solvent perturbation spectra of the BPTI species shows a progressive decrease in the ratio as refolding occurs (Table IV). It seems that in the case of ribonuclease the inaccessible tyrosines contribute significantly to the spectrum leading to a decrease in the ratio of peak heights. Furthermore, this also seems to be the case for the BPTI intermediates since the peak ratios given in Table IV are less than the ratio calculated for the spectrum of either R or Gly-Tyr-Gly.

We now suggest a correction for the perturbation spectra that appears to eliminate contributions by inaccessible tyrosines. This correction is empirical but does dramatically improve the agreement between the comparison spectra and the

perturbation spectra of the BPTI intermediates regardless of the model chosen for 100% solvent exposure. Furthermore, when this correction is applied to the perturbation spectrum of ribonuclease, the fractional exposure that is obtained is the same as when the solvent accessibility of the three accessible tyrosines was measured from the experimentally derived spectrum of those three tyrosines.

The correction is as follows: The ratio of the long to the short tyrosine wavelength peaks (Table IV) for a given BPTI intermediate is divided by the same ratio calculated for either of the two reference spectra. This value is then multiplied by the fractional tyrosine exposure previously calculated, i.e., the values in columns two and three of Table II. These corrected values are given in Table IV and compared there to the values calculated from the comparison spectra. As can be seen in Table IV the agreement between the two types of spectroscopy is much improved after the correction is applied, regardless of the model chosen for 100% solvent exposure. Excluding the values calculated for N^{SCM}_{SCM} and (30–51, 5–55), the average difference in the calculations for the two types of spectroscopy for a given reference model is 4%. When this correction was applied to the perturbation spectrum of ribonuclease using the *N*-acetyl-L-tyrosine ethyl ester spectrum as the model for 100% solvent exposure, a value of 36.6% tyrosine solvent exposure was calculated. This compared to a value of 35.2% derived directly (with no correction) from the spectra of the three accessible tyrosines of ribonuclease and the model tyrosine.

Finally, when the comparison is made between the experimental values determined from the spectra of native BPTI and the static accessible surface area determined from the crystalline structure of BPTI, the agreement between the three measurements is surprisingly close. If only the phenol ring is considered, i.e., the chromophore itself, the theoretical calculation gives a value of 31% solvent exposure, while the spectral measurements indicate 25%–36% solvent exposure for the tyrosines depending on the choice of reference compound.

Whether the absolute numerical values derived from difference spectroscopy or the correction that we have applied to the perturbation spectra have importance in the examination of a protein's solution conformation is not yet apparent. However, as calculations of aromatic amino acid solvent accessibility often are measurements in the study of proteins and the values derived from these measurements are often viewed as important, perhaps further study of solvent perturbation spectroscopy is called for in the manner similar to that chosen by Brandts and Kaplan for ribonuclease, as is an examination of our suggested correction.

Differences in the Spectra of the Three-Disulfide-Bond Proteins and the Two-Disulfide-Bond Proteins. Both comparison and perturbation spectroscopy indicate that some relatively small tyrosine solvent accessibility changes ($\approx\pm 6\%$) occur upon reduction or reoxidation of cystine-14–38. However, while both types of spectroscopy indicate that the tyrosine solvent accessibilities of N^{SCM}_{SCM} or (30–51, 5–55) are slightly different than those found for native or refolded BPTI, the comparison spectra and the perturbation spectra of the four proteins yield conflicting indications as to whether the tyrosines are, on average, more or less exposed to solvent when cystine-14–38 is reduced.

At face value, the comparison spectra of N^{SCM}_{SCM} or (30–51, 5–55) indicate that their tyrosines are slightly less exposed to solvent than are those of native BPTI, since the comparison spectra have positive values in the tyrosine wavelength region. (The statement is also true if the words "refolded BPTI" are substituted for "native BPTI" since the comparison spectrum of refolded BPTI indicated no differences in the degree of tyrosine solvent exposure between native and refolded BPTI.) On the other hand, the perturbation spectra of N^{SCM}_{SCM} or (30–51, 5–55) in contrast to the spectra of native or refolded BPTI indicate that, on the average, the tyrosines of the two-disulfide-bond proteins are slightly more exposed to solvent.

(Even without the use of our proposed correction for the perturbation spectra, in order for all four perturbation spectra to have approximately the same $\Delta A/A_{\lambda_{max}}$ value at the long-wavelength peak while the spectra of the two-disulfide-bond proteins show smaller $\Delta A/A_{\lambda_{max}}$ values at the shorter wavelength peak compared to the spectra of native or refolded BPTI, there must be a direct increase in the exposure of the tyrosines after cystine-14–38 is reduced. The unequal peak ratios found for the spectra of the two-disulfide-bond and the three-disulfide-bond proteins cannot be due solely to a decreased contribution of inaccessible tyrosines in N^{SCM}_{SCM} or (30–51, 5–55) since that should lead not only to an increase in the peak ratio but also to an absolute decrease in the value of $\Delta A/A_{\lambda_{max}}$ at the long-wavelength peak. This is not found.) Thus, while both types of spectroscopy indicate that small changes occur in the tyrosine solvent accessibilities of approximately 5%–9% when cystine-14–38 is reduced or reoxidized, the two types of spectroscopy indicate conflicting trends as to the extent of solvent exposure. Of all the BPTI intermediates, only N^{SCM}_{SCM} and (30–51, 5–55) (which have identical disulfides and are virtually interchangeable species) have comparison and perturbation spectra which indicate conflicting degrees of tyrosine solvent exposures compared to native BPTI.

If native BPTI and N^{SCM}_{SCM} were proteins whose structures were poorly understood, then this discussion would end here. However, as noted in the introduction, native BPTI and N^{SCM}_{SCM} are two of the most intensely studied of all proteins. More specifically, since detailed studies of the tyrosines of these two proteins have been made by methods other than difference spectroscopy, we feel that it is possible to offer an explanation that could resolve the conflict arising from the face value interpretations of the perturbation and comparison spectra of the four proteins. This proposed explanation is speculative and is offered, at least in part, as an incentive for others to use proteins with well-defined structures to further apply the technique of difference spectroscopy. Note that the following uses what has already been determined about the environments of the tyrosines of native BPTI and N^{SCM}_{SCM} to interpret the difference spectra rather than using difference spectroscopy to interpret the changes in the environments of the tyrosines upon reduction or reoxidation of cystine-14–38.

It has been shown that reduction followed by carboxyamidomethylation of cystine-14–38 alters the proton NMR resonances and therefore the environments of only two tyrosines, those at positions 10 and 35 (Snyder et al., 1976; Wagner et al., 1979a). After the modification, the proton NMR resonances associated with tyrosine-10 are similar to those of a model tyrosine compound indicating that this tyrosine is less perturbed by other portions of the protein and therefore probably more exposed to solvent. (Tyrosine-10 is in large part exposed to solvent in the native protein. See Table III.) Four individual resonances for the four ring protons have been assigned to tyrosine-35 in the NMR spectrum of native BPTI. Observation of four resonances indicates that tyrosine-35 is immobile on the NMR time scale and therefore this tyrosine must be nearly completely buried. Table III also indicates the relatively low degree of solvent accessibility of tyrosine-35. After reduction and alkylation of cystine-14–38, the NMR resonances of tyrosine-35 are slightly altered, but there are

still four resonances. Therefore, while the environment of this tyrosine is altered, the NMR evidence indicates that this tyrosine remains inaccessible to solvent.

It has also been shown that the hydroxyl group of tyrosine-35 is involved in a hydrogen bond with the peptide amide of cystine-38 in the crystalline protein and that this hydroxyl titrates at an abnormally high pH (pK = 11.0). Alkylation of cysteines-14 and -38 with the neutral carboxyamidomethyl group has no effect on the pK of the hydroxyl group of tyrosine-35. However, whether two negatively charged carboxymethyl groups affect the hydrogen bond formed between the amide of cysteine-38 and the hydroxyl of tyrosine-35 either directly or indirectly is as yet unknown.

Using the observations made by NMR spectroscopy, we presume that the apparent increased solvent accessibility of the tyrosines, which occurs when cystine-14–38 is reduced as indicated by the solvent perturbation spectra of N^{SCM}_{SCM} or (30–51, 5–55) in comparison to the spectra of native or refolded BPTI, is probably due primarily to an increased interaction between tyrosine-10 and ethylene glycol. Additional or altered contributions by tyrosine-35 to the perturbation spectra of N^{SCM}_{SCM} and (30–51, 5–55) are not ruled out, but when the reasoning outlined above is used, tyrosine-35 should not be the dominating factor since this residue does not appear to be exposed to solvent after reduction of the cystine. On the other hand, since the comparison spectra indicate less solvent exposure after reduction and alkylation, the presumed increased solvent exposure of tyrosine-10 should not be the dominating factor in these spectra. Instead, we propose that the introduction of the two negative carboxymethyl groups at cysteines-14 and -38 influences the strength of the hydrogen bond between the hydroxyl of tyrosine-35 and the amide of cysteine-38, leading to a small red shift in the spectra of N^{SCM}_{SCM} and (30–51, 5–55). Opposing this influence we would expect a negative contribution by tyrosine-10 to the comparison spectra of N^{SCM}_{SCM} and (30–51, 5–55). However, as the comparison spectra are not negative, we suspect that the contribution of tyrosine-10 to these spectra is considerably weaker than the contribution of tyrosine-35.

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