Circular Dichroism Spectroscopy of Bovine Pancreatic Trypsin Inhibitor and Five Altered Conformational States. Relationship of Conformation and the Refolding Pathway of the Trypsin Inhibitor†

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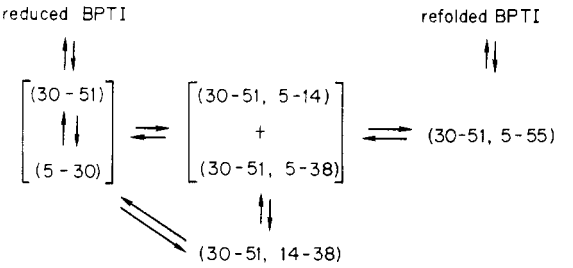
ABSTRACT: As part of a conformational study of the pathway of unfolding and refolding of bovine pancreatic trypsin inhibitor that accompanies breakage and formation of its three disulfide bonds, circular dichroism spectra have been measured for several limiting conformational states: native and refolded, with the three correct disulfide bonds; the (30–51, 5–55) two-disulfide species trapped during unfolding and refolding, which have a stable nativelike conformation; the fully reduced protein, with no disulfide bonds. Refolded protein with the three correct disulfide bonds has been found to be slightly different from the native protein; this conformational difference could be removed by gently heating the refolded protein. The same difference appears to be present between the two-disulfide intermediates, lacking the 14–38 disulfide bond, produced during unfolding and refolding. The conformational difference appears to be introduced at an early stage of refolding. The fully reduced protein, with no disulfides, exists as a flexible polypeptide chain with no detectable fixed conformation. The near-ultraviolet portions of the spectra are resolved into probable contributions by tyrosine, disulfide, and phenylalanine side-chain electronic transitions. The probable contributions to the native protein spectrum by tyrosines were also elucidated by observing the spectral shifts caused by their ionization at pH 12.5, where the folded conformation is maintained. The rotational strengths of the isolated transitions provide a measure of conformational flexibilities for the chromophores. Resolution of the far-ultraviolet spectrum of the native protein into contributions of its known secondary structures was not successful.

Bovine pancreatic trypsin inhibitor (BPTI)[1] is a relatively small protein with a single polypeptide chain of 58 amino acid residues. The protein has three disulfide bonds linking cysteines-14–38, -30–51, and -5–55, four tyrosines at positions 10, 21, 23, and 35, and four phenylalanines at positions 4, 22, 33, and 45 (Kassell & Laskowski, 1965; Kassell et al., 1965; Anderer & Hörnle, 1966). The crystal structure (Huber et al., 1971) has been refined to 1.5 Å (Deisenhofer & Steigemann, 1975). A model of native BPTI is seen in Figure 1. BPTI has become an attractive model for the experimental and theoretical study of protein conformation, due in part to its small size, highly refined crystal structure, and an extreme resistance to denaturation (Vincent et al., 1971; Karplus et al., 1973; Brunner & Holz, 1975; Wüthrich & Wagner, 1979).

BPTI has also been used successfully in an examination of the refolding process from the reduced and denatured state to the reoxidized, refolded, and biochemically active inhibitor [reviewed by Creighton (1978, 1980a)]. The pathway of refolding has been elucidated by trapping in a stable form the disulfide bonds in the limited number of kinetically important intermediates that accumulate during unfolding and refolding. The trapped intermediates have been isolated, their disulfides identified, and their kinetic roles characterized (Creighton, 1974, 1975a, 1977a,b).

The refolding pathway can be represented as



where the intermediates are represented by their disulfide bonds.

Now that the refolding pathway has been clarified in terms of disulfide formation, the question as to the conformational forces guiding the pathway can be addressed by examining the conformations of each refolding species. An immunochemical study of the binding of each refolding intermediate to antibodies raised against the native and against the denatured protein has been reported (Creighton et al., 1978). Near-UV difference spectroscopy has been used to examine

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[1] Abbreviations used for proteins: BPTI, bovine pancreatic trypsin inhibitor, irrespective of the states of the disulfide bonds. The protein, prior to unfolding and reduction, is referred to as native BPTI. The protein is referred to as refolded BPTI after reoxidation and refolding. R is the fully reduced, denatured protein with the six cysteines carboxyamidomethylated. N^{SCM}_{SCM} is formed from native BPTI by selective reduction of the 14–38 disulfide. Other forms of BPTI are designated by the residue numbers which are involved in disulfide bonds. For example, a one-disulfide-bond species with the 30–51 disulfide is denoted as (30–51). All thiol groups have been carboxymethylated. Other abbreviations: $A_{\lambda_{max}}$, the absorbance at the wavelength of maximal absorption in the near-ultraviolet; CD, circular dichroism; $\epsilon_{\lambda_{max}}$, the extinction coefficient at the wavelength of maximal absorption in the near-ultraviolet; Gdn·HCl, guanidine hydrochloride; Pipes, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); $[\theta]$, mean residue ellipticity; Tris, tris(hydroxymethyl)aminomethane; UV, ultraviolet; θ_0, λ_0, and Δ_0, the maximal ellipticity, the wavelength position of θ_0, and the band half-width of an electronic transition, respectively.

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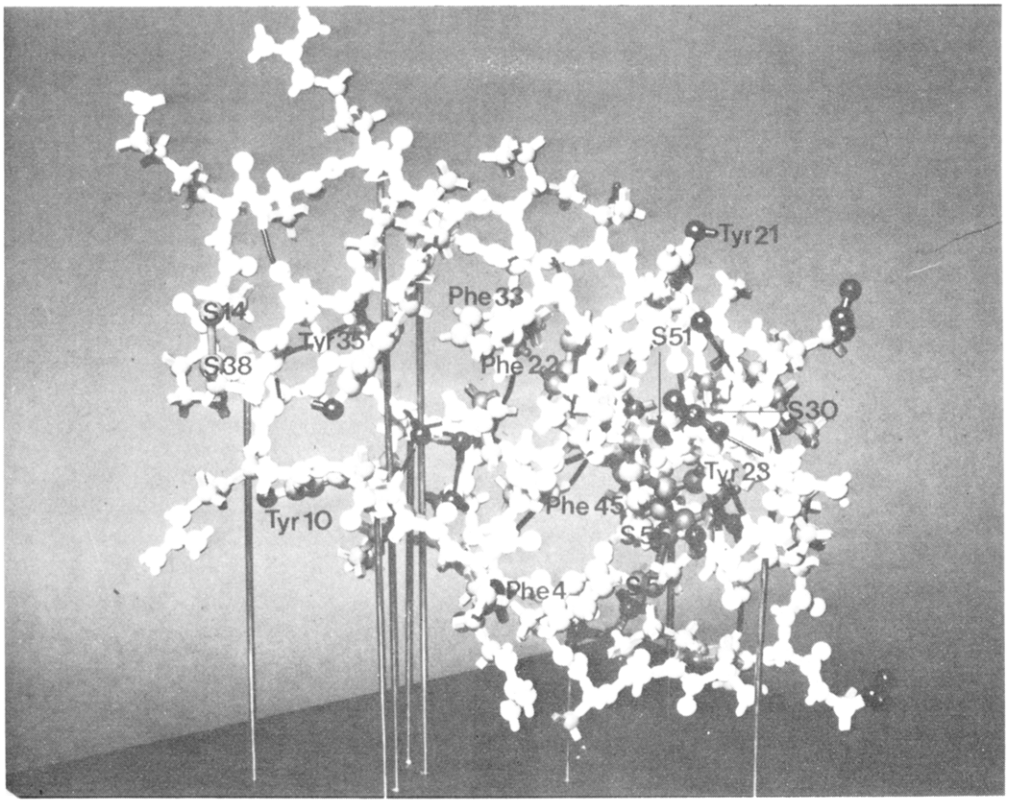

FIGURE 1: Model of native BPTI. The tyrosines, phenylalanines, and disulfides are identified.

the solvent accessibilities of the aromatic amino acids in each state (Kosen et al., 1980). A comparison of native and refolded BPTI has been made by proton NMR spectroscopy (States et al., 1980). A circular dichroism study of each refolding species has also been made (Kosen, 1978). This paper reports the results of the CD study for five of the refolding conformations of BPTI, including native BPTI, refolded BPTI, the denatured protein with the six cysteines carboxyamidomethylated (R), and two forms of BPTI with the disulfides 30–51 and 5–55. Of the last two, (30–51, 5–55) is formed during refolding, and the other, N^{SCM}_{SCM}, is formed by selective reduction of the native 14–38 disulfide, i.e., as the first step in unfolding. In addition, the CD spectrum of native BPTI at alkaline pH has been examined to clarify the strength of the tyrosine transitions. The effects of temperature and Gdn·HCl on the spectrum of R were measured to see if they produced unfolding of any ordered conformation.

Circular dichroism has been an important technique in probing the conformations of the various BPTI species. Because the magnitudes of the different CD spectra [reported here and for the other refolding intermediates by P. A. Kosen, T. E. Creighton, and E. R. Blout (unpublished experiments)] varied greatly, definitive differences in the tyrosine and disulfide side-chain environments were determined with the use of curve resolution.

Materials and Methods

Materials. Ultrapure guanidine hydrochloride (Gdn·HCl) was a product of Schwarz/Mann Biochemicals. Piperazine-*N*-*N*′-bis(2-ethanesulfonic acid) (Pipes), tris(hydroxymethyl)aminomethane (Tris), and *trans*-4,5-dihydroxy-1,2-dithiane were obtained from Sigma Chemical Co.

Proteins. Native BPTI (Trasylol, lot GOS 746/31) was a gift of Bayer AG. All of the refolding species were prepared as previously described (Creighton, 1975a,b, 1977a). All of the intermediates were isolated as homogeneous species except for (30–51, 5–14) and (30–51, 5–38), which were obtained as a mixture of comparable proportions of the two species.

The lyophilized proteins were stored at 4 °C until used. Unless stated otherwise, the proteins were dissolved in 6.0 mM Pipes, pH 6.8, and the solutions filtered with a Millipore HATF 01300 filter before their spectra were recorded.

Estimation of Protein Concentrations. The solution concentrations of the BPTI folding intermediates were routinely determined by using a Cary 14 or 15 spectrophotometer and Beer's law, assuming $\epsilon_{\lambda_{max}}$ in the near-UV as 5720 L/(cm·mol), which is the value at 277.5 nm (λ_{max}) for native BPTI (Kassell, 1970). The accuracy of this method for all BPTI species was demonstrated experimentally in two ways. The first measured the total change in $A_{\lambda_{max}}$ during refolding. Native BPTI was reduced and denatured overnight (Creighton, 1975a). The denaturants were removed by gel filtration in 0.01 M HCl, and the near-UV spectrum of the fully reduced protein was recorded. The reduced protein was then refolded by dilution into 0.1 M Tris-HCl, pH 8.0, 0.2 M KCl, and 1.0 mM EDTA, with 1.0 mM trans-4,5-dihydroxy-1,2-dithiane as an oxidant, or into a similar buffer, lacking EDTA and *trans*-4,5-dihydroxy-1,2-dithiane, for air oxidation. The spectrum was then recorded at various times until no changes were seen. Reference blanks were obtained by dilution of 0.01 M HCl into the appropriate buffer. Cuvette path lengths and dilution factors were chosen so that $A_{\lambda_{max}}$ remained between 0.7 and 1.3 absorbance units. The change in $\epsilon_{\lambda_{max}}$ ($\Delta\epsilon$) was determined by direct comparison of $A_{\lambda_{max}}$ before and after air oxidation, or by solving for $\Delta\epsilon$ in the equation:

$$A^{R}_{\lambda_{max}}/A^{\text{refolded BPTI}}_{\lambda_{max}} = (5720 - \Delta\epsilon)/(5720 - 3 \times 260)$$

when *trans*-4,5-dihydroxy-1,2-dithiane was used as the oxidant. The extinction coefficient is 260 at 277.5 nm for *trans*-4,5-dihydroxy-1,2-dithiane (Cleland, 1964), and it was assumed that three molecules of *trans*-4,5-dihydroxy-1,2-dithiane were reduced concomitant with the refolding of one protein mole-

Table I: Comparison of the Concentrations of Solutions of the BPTI Folding Species by the Method of Lowry et al. (1951) and by Near-UV Spectroscopy

BPTI folding species	concn of stock solution (mg/mL) Lowry method	UV spectroscopy	difference in the two measurements (%)
refolded BPTI	0.605	0.599	1.0
	0.574	0.589	3.0
N^{SCM}_{SCM}	1.155	1.192	3.0
	1.234		4.0
(30–51, 5–55)	0.370	0.379	2.0
	0.371		2.0
R	0.657	0.654	0.0
	0.645		1.0
(30–51)	0.613	0.580	6.0
	0.584		1.0
(5–30)	0.632	0.616	3.0
	0.593		3.0
(30–51, 5–14) + (30–51, 5–38)	0.872	0.855	2.0
	0.915		7.0
(30–51, 14–38)	0.621	0.649	4.0
	0.671		3.0

cule. Complete refolding has been shown to occur under these conditions and was assumed when the spectrum no longer changed; it was then very similar to that of native BPTI. Each refolding experiment was performed twice. With *trans*-4,5-dihydroxy-1,2-dithiane as the oxidant, $\Delta\epsilon$ equalled 363 ± 26 L/(cm·mol); the value was 255 ± 76 L/(cm·mol) for the air oxidation experiments. Therefore, on folding, $\epsilon_{\lambda_{max}}$ increases by not more than 5.4% ± 1.6%.

In the second procedure, the concentrations of the refolding intermediates were determined by the method of Lowry et al. (1951) with native BPTI as the protein standard. These determinations were compared to concentration measurements made from the UV spectra of stock solutions used for the Lowry assays. The results of Table I demonstrate agreement within 5%.

Circular Dichroism Measurements. Spectra were recorded with a Cary 60 spectropolarimeter and a Model 6001 CD attachment, using cuvettes of 0.01–2.0-cm path lengths. Each reported spectrum is the average of at least two separate protein samples; the spectrum of each was recorded at least 3 times. The spectra are expressed in mean residue ellipticity (deg·cm^2/dmol). Mean residue concentrations were calculated by using the following molecular weights and mean residue weights, respectively: for native and refolded BPTI, 6513 and 112.3; for N^{SCM}_{SCM} and (30–51, 5–55), 6627 and 114.3; for R, 6855 and 118.2.

Curve resolution, i.e., the reduction of a near-UV CD spectrum into a *minimum number* of Gaussian curves representing chromophoric electronic transitions, was performed by a computer program developed by R. G. Hammonds, Jr., of Vanderbilt University and modified by J. Brunelle of Harvard Medical School to the specifications of a Digital Equipment Corp. PDP-11/70 computer running under the CULC UNIX system.

Results

CD Spectra of Native BPTI and Refolded BPTI. The spectra of these three-disulfide-bond, biochemically active proteins, shown in Figure 2, are not identical. The differences in the two spectra are small (~6% at the near- and far-UV

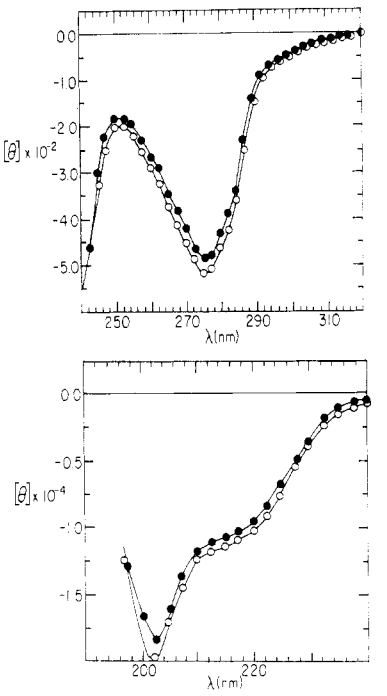

FIGURE 2: CD spectra of native BPTI (○) and refolded BPTI (●) in 6.0 mM Pipes, pH 6.8. (Top) Near-UV portion of the spectrum; for native BPTI, $[\theta]_{275} = -520 \pm 9$ deg·cm^2/dmol (mean ± SE); for refolded BPTI, $[\theta]_{275} = -490 \pm 6$ deg·cm^2/dmol. (Bottom) Far-UV portion of the spectrum: for native BPTI, $[\theta]_{202.5} = -19\,800 \pm 250$ deg·cm^2/dmol; for refolded BPTI, $[\theta]_{202.5} = -18\,450 \pm 110$ deg·cm^2/dmol.

minima) but reproducible and indicate differences in conformation.

The overall features of the two spectra are the same. In the near-UV, there are *very* slight shoulders near 260, 265, and 282.5 nm, a minimum at 275 nm, and gradually decreasing ellipticity above 290 nm. With model compound spectra as guides (Horwitz et al., 1969, 1970; Kahn, 1972; Holladay et al., 1974, 1976; Strickland, 1974; Holladay & Puett, 1976), the slight shoulders near 260 and 265 nm were assigned to phenylalanine transitions and the minimum at 275 nm and the slight shoulder near 282.5 nm to tyrosine transitions, and the ellipticity above 290 nm is indicative of negative disulfide transitions.

Both spectra are also characterized by a minimum at 202.5 nm and a shoulder between 215 and 225 nm. For reasons detailed later, these features were not assigned to peptide bond transitions associated with specific secondary structures. The spectrum of native BPTI is identical with that reported by Quast et al. (1975).

Heat Annealing of Refolded BPTI. The CD spectrum of refolded BPTI became identical with that of native BPTI when neutral solutions were heated to 50 or 70 °C for 1 h and then cooled to room temperature; 70 °C, however, is below the temperature at which denaturation of native BPTI can be observed. Heat thus alters the conformation of refolded BPTI so that it apparently becomes identical with that of native BPTI.

CD Spectra of N^{SCM}_{SCM} and (30–51, 5–55). These two species have the same two disulfide bonds and hold the same relative positions on the unfolding–refolding pathway of BPTI (Creighton, 1977a). While the overall shapes of their CD spectra are the same (Figure 3), the magnitudes of the two spectra differ by about 5–7%, with the spectrum of (30–51, 5–55) being the less intense. Thus, the conformations of N^{SCM}_{SCM} and (30–51, 5–55) are unlikely to be identical.

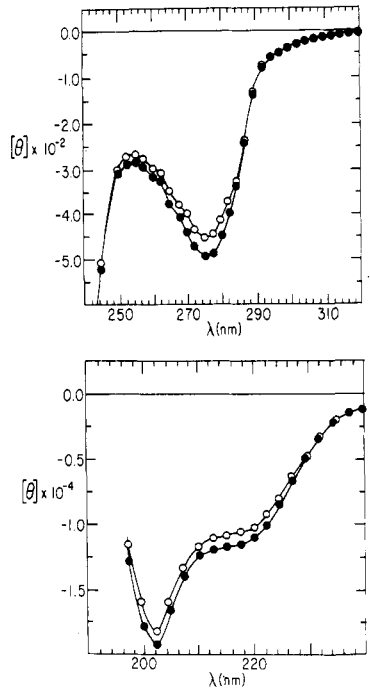

FIGURE 3: CD spectra of N_{SCM}^{SCM} (●) and (30–51, 5–55) (○) in 6.0 mM Pipes; pH 6.8. (Top) Near-UV portion of the spectrum: for N_{SCM}^{SCM}, $[\theta]_{275} = -490 \pm 8$ deg·cm²/dmol; for (30–51, 5–55), $[\theta]_{275} = -450 \pm 10$ deg·cm²/dmol. (Bottom) Far-UV portion of the spectrum: for N_{SCM}^{SCM}, $[\theta]_{202.5} = -19\,350 \pm 115$ deg·cm²/dmol; for (30–51, 5–55), $[\theta]_{202.5} = -18\,350 \pm 160$ deg·cm²/dmol.

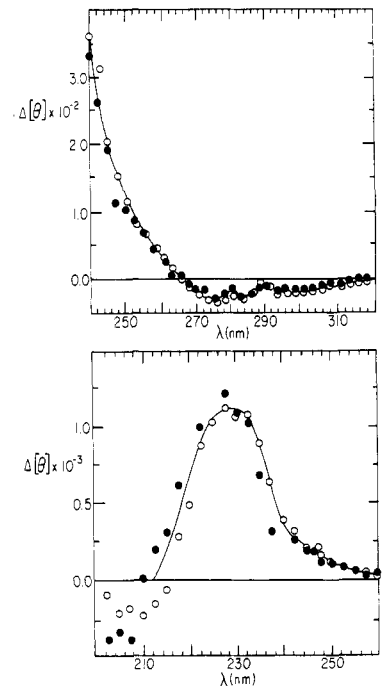

FIGURE 4: Calculated CD difference spectra: native BPTI minus N_{SCM}^{SCM} (●); refolded BPTI minus (30–51, 5–55) (○). Values were calculated every 2.5 nm.

Comparison of the Three- and Two-Disulfide-Bond Proteins. Native BPTI and N_{SCM}^{SCM} have very similar conformations, although the latter lacks the 14–38 disulfide (Vincent et al., 1971; Brunner et al., 1974; Snyder et al., 1976; Creighton et al., 1978; Wagner et al., 1979a,b); their CD spectra reflect this similarity. Changes in the near-UV include more noticeable shoulders near 260 and 265 nm, increased negative ellipticity near 250 nm, and a decrease in ellipticity at 275 nm in the spectrum of N_{SCM}^{SCM}. The major alteration in the far-UV is an increase and altered shape in the 215–225-nm shoulder; the minimum at 202.5 nm is basically unaltered. Correlation of the spectral with the known structural alterations will be made under Discussion.

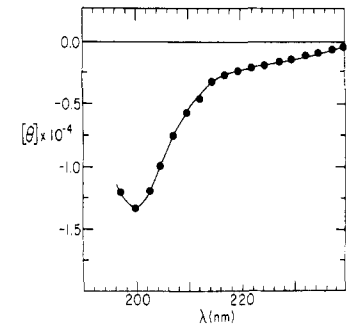

FIGURE 5: Far-UV CD spectrum of R in 6.0 mM Pipes, pH 6.8. $[\theta]_{200} = -13\,250 \pm 300$ deg·cm²/dmol.

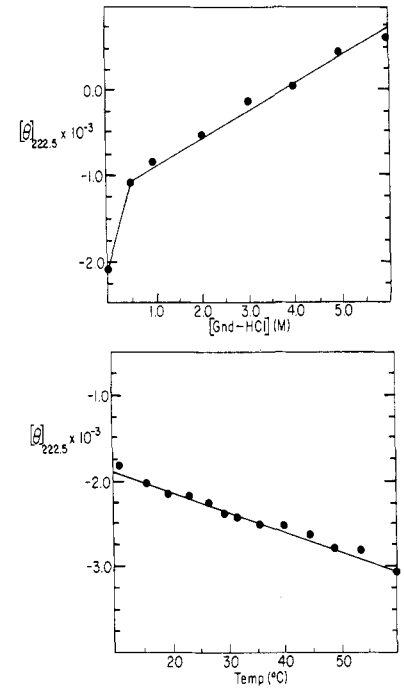

FIGURE 6: (Top) Dependence of $[\theta]_{222.5}$ on [Gdn·HCl] for R in 6.0 mM Pipes, pH 6.8. (Bottom) Dependence of $[\theta]_{222.5}$ on temperature for R in 6.0 mM Pipes, pH 6.8. Temperature was maintained by circulating a water–ethylene glycol mixture through a hollow brass cuvette compartment insulated with cork. A temperature probe was directly inserted into the cuvette.

The spectral changes upon loss of the 14–38 disulfide bond can also be illustrated by the CD difference spectrum, native BPTI minus N_{SCM}^{SCM} (Figure 4). The same comparison of refolded BPTI and (30–51, 5–55) is also shown; these two CD difference spectra are virtually identical.

CD Spectrum of R. The spectrum of R (Figure 5) is characteristic of denatured proteins (Fasman et al., 1970). The single minimum at 200 nm, the lack of characteristic ellipticity between 210 and 240 nm, and negative ellipticity throughout the measurable region (to 190 nm which is not shown) all indicate the absence of α helices and β sheets (Greenfield & Fasman, 1969; Chen et al., 1974). The region above 240 nm is featureless and not shown.

The effects of Gdn·HCl and temperature on the spectrum of R were measured. The results of these experiments are shown as titration curves in Figure 6 where the value of $[\theta]_{222.5}$ was plotted against increasing Gdn·HCl concentrations and against temperature. Except for a break between 0.0 and 1.0

Table II: Comparison of Calculated Secondary Structure Estimates for Native BPTI[a] and Structure Estimates from Crystallographic Studies[b]

secondary structure	calcd amount of secondary structure[c] from the model CD spectra of: Greenfield and Fasman, constrained	Greenfield and Fasman, unconstrained[d]	Chen et al., constrained	Chen et al., unconstrained[d]	amount of secondary structure[e] determined by crystallography
helix	33	21	35	30	17
β sheet	2	40	−19	43	33–40[f]
aperiodic structure	65	97	84	122	50–43

[a] The CD reference spectra of Greenfield & Fasman (1969) and Chen et al. (1974) were used. [b] Crystallographic studies of Huber et al. (1971) and Deisenhofer & Steigemann (1975). [c] Calculated values are expressed as percentages. [d] Under constrained conditions, the sum of the amounts of secondary structures must equal 100%. Under unconstrained conditions, the sum of the amounts of secondary structures can equal any value. [e] An α helix is formed by residues 47–56, and a β sheet by residues 16–36. [f] The values of the β sheet and aperiodic structure are expressed as ranges since the β-sheet size has been calculated by including and excluding the turn made by residues 24–27 which connect the two strands of the sheet.

Table III: Tentative Assignments and Parameters of the Resolved Gaussian Bands in the Spectra of Native BPTI, Refolded BPTI, N^{SCM}_{SCM}, and (30–51, 5–55)[a]

tentative assignment	native BPTI λ_0	native BPTI Δ_0	native BPTI θ_0	refolded BPTI λ_0	refolded BPTI Δ_0	refolded BPTI θ_0	N^{SCM}_{SCM} λ_0	N^{SCM}_{SCM} Δ_0	N^{SCM}_{SCM} θ_0	(30–51, 5–55) λ_0	(30–51, 5–55) Δ_0	(30–51, 5–55) θ_0
Tyr												
0-0 (1)[b]	282.3	6.3	−271	282.1	6.4	−254	282.8	6.1	−243	282.5	6.2	−228
0 + 800 (2)	275.7	3.9	−163	275.7	3.8	−136	276.2	4.0	−147	276.4	3.4	−106
0 + 2(800) (3)	270.1	6.0	−191	270.3	5.8	−189	270.6	6.0	−167	271.2	5.5	−161
0 + 3(800)[c] (4)	264.1	4.7	−45	264.1	4.7	−45	264.8	2.6	−41	264.8	2.6	−41
-S-S- (5)	264.3	27.0	−229	264.8	29.0	−214	262.9	24.9	−276	262.3	26.3	−270
Phe												
0 + 930 (6)	259.7	2.5	−33	259.4	2.5	−33	259.1	2.6	−26	259.1	2.6	−26

[a] Assignments are based on studies by Horwitz et al. (1969, 1970), Kahn (1972), Holladay et al. (1974, 1976), and Holladay & Puett (1976). λ_0 and Δ_0 are expressed in nanometers; θ_0 is expressed as deg·cm²/dmol on a mean residue basis. [b] Values in parentheses correspond to band numbers in Figures 7 and 8. [c] This band, in all four spectra, may also contain a contribution by a phenylalanine 0–0 transition.

M Gdn·HCl, both titration curves are linear. The absence of sigmoidicity in the titration curves indicates that R does not have a stable conformation.

Curve Resolution of the Spectra. Two types of curve resolution were attempted to gain a better understanding of the protein conformations for all folding intermediates. Using a linear least-squares analysis and either of two sets of model secondary structure CD spectra [i.e., the poly(L-lysine) spectra recorded by Greenfield & Fasman (1969) or composite spectra calculated from the crystal structures and CD spectra of five proteins (Chen et al., 1974)], we calculated the amounts of secondary structures from the far-UV CD spectra. Because the calculated values for native BPTI (Table II) differed strongly with the known amounts of secondary structures (Huber et al., 1971; Deisenhofer & Steigemann, 1975), this method was abandoned; no attempt was made to assign secondary structure transitions to the far-UV regions of any of the spectra.

A more useful method involved resolving each near-UV CD spectrum into a minimum number of Gaussian curves, which when summed, equalled, as nearly as possible, the experimental spectrum. This technique, its limitations, and the rationale for its use are briefly outlined under Appendix. Each Gaussian curve was tentatively assigned to a tyrosine, disulfide, or phenylalanine electronic transition (using as guides model compound spectra) and has an associated magnitude (θ_0), wavelength position (λ_0), and band half-width (Δ_0). The rotational strengths [$R = (1.234 \times 10^{-42})\theta_0\Delta_0/\lambda_0$ cgsu] for a group of chromophoric transitions can be compared to those of model compound spectra and are indications of the relative flexibility or rigidity of those chromophores (Strickland et al., 1970, 1972; Kahn, 1972; Takagi & Ito, 1972; Holladay et al., 1974, 1976; Strickland, 1974; Holladay & Puett, 1976).

Resolution of the native BPTI spectrum is shown in Figure 7, with tentative band assignments and parameters given in Table III. A comparison is made in Table IV between the rotational strengths of the experimental transitions and those of certain model compounds (Kahn, 1972; Holladay et al., 1974, 1976; Holladay & Puett, 1976). An important point emerging from the comparison is that the rotational strengths of the presumed tyrosine and disulfide transitions of the BPTI spectrum are much stronger than those of aqueous model compounds. (This observation is valid even if the resolution is not completely correct, since it reflects the overall intensity of the near-UV CD spectrum of native BPTI.)

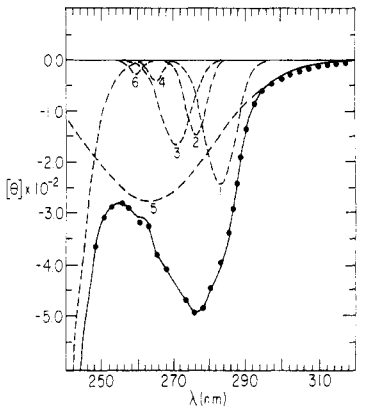

FIGURE 7: Resolution of the near-UV portion of the native BPTI spectrum (Figure 2): experimental values (●); resolved Gaussian bands (- - -); sum of the Gaussian bands (—). The band at the lowest wavelengths was included only to completely fit the spectrum; no assignment was made for this band. All other bands are numbered and correspond to the numbers in Table III.

The refolded BPTI spectrum was resolved by altering the transitions of the native BPTI spectrum to fit the former. As seen in Table III, no large alterations are necessary; the principal changes involved tyrosine transitions. (The resolved refolded BPTI spectrum is not shown.)

Table IV: Molar Rotational Strengths (cgs Units) and Comparison with the Molar Rotational Strengths from the Resolutions of Some Model Compound Spectra[a]

tentative assignment[b]	native BPTI		refolded BPTI		N_{SCM}^{SCM}		(30–51, 5–55)	
	$R_0 \times 10^{42}$	R_0/R_m	$R_0 \times 10^{42}$	R_0/R_m	$R_0 \times 10^{42}$	R_0/R_m	$R_0 \times 10^{42}$	R_0/R_m
Tyr[c]								
0–0	−108.0	5.9	−103.0	5.7	−93.9	5.2	−89.6	4.9
0 + 800	−41.2	7.5	−33.6	6.1	−38.0	6.9	−23.3	4.2
0 + 2(800)	−75.8	7.6	−72.7	7.3	−66.1	6.6	−58.2	5.8
0 + 3(800)	−14.2	8.1	−14.2	8.1	−7.2	4.1	−7.2	4.1
		(7.0)[f]		(6.4)[f]		(6.2)[f]		(5.0)[f]
–S–S–[d]	−557.0		−560.0		−935.0		−970.0	
		3.2		3.2		5.4		5.6
		2.2		2.2		3.7		3.8
		0.62		0.62		1.0		1.1
Phe[e]								
0 + 930	−5.7	4.9	−5.6	4.8	−4.6	4.1	−4.6	4.1

[a] $R_0 = 58/[n(1.234 \times 10^{-42})(\theta_0\Delta_0/\lambda_0)]$ cgsu, where n is the number of chromophores, i.e., four tyrosines, four phenylalanines, or two or three disulfides. [b] Tentative assignments are as in Table III. [c] R_m is an averaged molar rotational strength for the resolved CD transitions of glycyl-L-tyrosylglycine in 0.02 M Tris-HCl, pH 7.0; 6.0 M Gdn·HCl and 0.02 M Tris-HCl, pH 7.0; and 80% ethylene glycol and 0.02 M Tris-HCl, pH 7.0 (Holladay & Puett, 1976). The R_m values of the three model spectra are of similar intensities. [d] The R_0/R_m values were calculated by using the molar rotational strength of the longest wavelength resolved band for three different model spectra. From top to bottom: L-cystine, 1.0 M KF, and 0.02 M Tris-HCl, pH 7.5 (Holladay et al., 1976); L-cystine, pH 5.06–5.9 (Kahn, 1972); hexagonal L-cystine KBr disk (Kahn, 1972). [e] R_m is an averaged molar rotational strength for the resolved CD transitions of glycyl-L-phenylalanylglycine in 0.01 M KCl and 0.002 M sodium phosphate, pH 7.5, and in 80% ethylene glycol, 0.01 M KCl, and 0.002 M sodium phosphate, pH 7.5 (Holladay et al., 1974). The R_m values of the two model compound spectra are of similar intensities. [f] The average R_0/R_m value for the 0–0, 0 + 800, and 0 + 2(800) transitions.

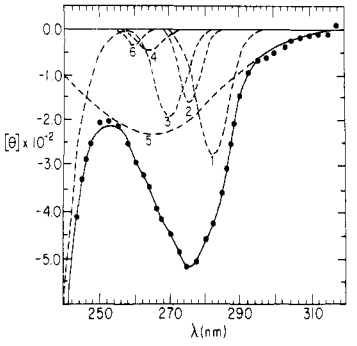

FIGURE 8: Resolution of the near-UV portion of the N_{SCM}^{SCM} spectrum (Figure 3): experimental values (●); resolved Gaussian bands (---); sum of the Gaussian bands (—). The band at the lowest wavelengths was included only to completely fit the spectrum; no assignment was made for this band. All other bands are numbered and correspond to the numbers in Table III.

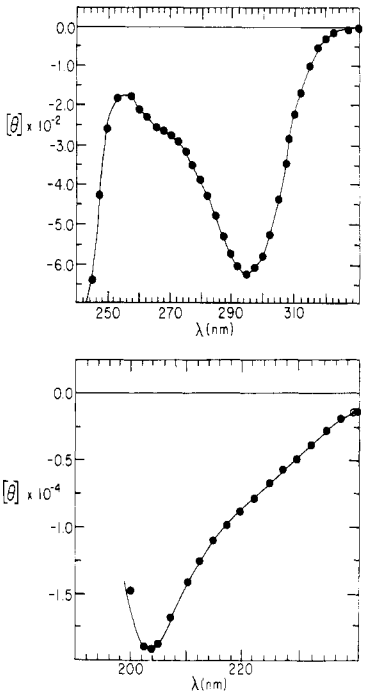

FIGURE 9: CD spectrum of native BPTI in 6.0 mM sodium phosphate, pH 12.5. Samples were prepared by dilution of equal volumes of native BPTI in H_2O and 12.0 mM sodium phosphate, pH 12.5. Concentrations were determined before dilution. (Top) Near-UV portion of the spectrum; $[\theta]_{295} = -620 \pm 10$ deg·cm²/dmol. (Bottom) Far-UV portion of the spectrum; $[\theta]_{203} = -19\,250 \pm 300$ deg·cm²/dmol.

Resolutions were also performed on the spectra of N_{SCM}^{SCM} and (30–51, 5–55). The resolved spectrum of N_{SCM}^{SCM} is shown in Figure 8, and the band assignments etc. of both N_{SCM}^{SCM} and (30–51, 5–55) are found in Table III. Comparison of the tyrosine and disulfide transitions in the spectra of native BPTI and N_{SCM}^{SCM} (Table IV) indicated a large increase in the disulfide contributions and a significant decrease in the tyrosine contributions upon the 14–38 disulfide reduction. This disagrees with other evidence showing that the environments of the tyrosines and remaining disulfides are almost unaffected by the selective reduction (Brunner et al., 1974; Snyder et al., 1976; Wagner et al., 1979a,b).

CD Spectrum of Native BPTI at pH 12.5. The spectrum of native BPTI, pH 12.5 (Figure 9), was recorded to clarify the nature of the tyrosine transitions. At pH 12.5, native BPTI maintains a conformation which is almost identical with that existing at neutral pH, but with the tyrosines fully or almost fully ionized (Sherman & Kassell, 1968; Vincent et al., 1971; Karplus et al., 1973; Snyder et al., 1975; Wagner & Wüthrich, 1975). A red shift of the tyrosine transitions from about 275 nm to above 290 nm, as occurs with model tyrosine compounds (Strickland, 1974; Ascoli et al., 1977), was expected upon ionization of the BPTI tyrosines, leaving any disulfide and phenylalanine transitions as the predominant features below about 280 nm. A new minimum indeed appeared above 290 nm at pH 12.5, and the significant ellipticity remaining in the region of 270 nm is likely due to disulfide transitions; the two shoulders assigned to phenylalanine transitions are also more obvious.

The far-UV region was also altered at the higher pH. The shoulder was lost, but the region between 225 and 240 nm increased in magnitude. The minimum shifted to 203 nm with unchanged intensity.

It was easier to identify spectral changes resulting from the pH shift by a CD difference spectrum between native BPTI

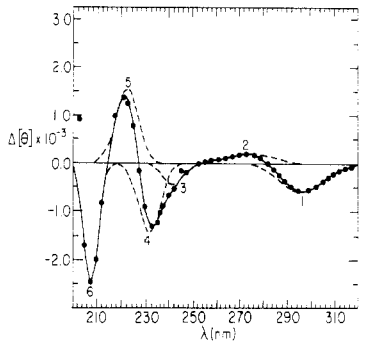

FIGURE 10: Calculated CD difference spectrum; native BPTI, pH 12.5, minus native BPTI, pH 6.8. Values were calculated every 2.5 nm; calculated values (●); resolved Gaussian bands (---); sum of the Gaussian bands (—). In regions where the resolved Gaussian bands and the sum of the bands overlap, only the sum of the bands (—) is drawn. Each band is numbered and corresponds to the number in Table V.

Table V: Tentative Assignments, Parameters, and Molar Rotational Strengths of the Resolved Gaussian Bands in the CD Difference Spectrum, Native BPTI, pH 12.5, Minus Native BPTI, pH 6.8[a]

Tyr transition	λ_0	Δ_0	θ_0	$R_0 \times 10^{42}$
L_b, pH 12.5 (1)[b]	297.0	13.1	−590	−465.2
L_b, pH 6.8 (2)	274.4	12.8	227	189.1
L_a, pH 12.5 (3)	241.6	6.1	−445	−201.0
L_a, pH 12.5 (4)	231.7	6.4	−1420	−699.8
L_a, pH 6.8 (5)	222.2	6.4	1550	799.3
B_b, pH 12.5 (6)	207.6	4.3	−2460	−912.5

[a] Assignments are based on studies by Holladay & Puett (1976). λ_0 and Δ_0 are expressed in nanometers; θ_0 is expressed in deg·cm²/dmol on a mean residue basis; $R_0 = 58/[(4)(1.234 \times 10^{-42})(\theta_0\Delta_0/\lambda_0)]$ cgsu. [b] Values in parentheses correspond to band numbers in Figure 10.

at pH 12.5 and 6.8 (Figure 10). This difference spectrum has been resolved, and the assigned bands are listed in Table V. There appear to be three sets of transitions, each including at least one negative and one positive band.

One set (L_b transitions) included the negative band centered at 297 nm and the positive at 274.4 nm. The former was assigned to the transitions of the ionized tyrosines and the latter to the tyrosine transitions lost upon ionization. The latter would be negative in the native BPTI spectrum. The rotational strength of the 274.4-nm differential band (189.1×10^{-42} cgsu/tyrosine)[2] is similar to the sum of the rotational strengths of the tyrosine transitions in the spectrum of N^{SCM}_{SCM} (-205.2×10^{-42} cgsu/tyrosine); both are smaller than the sum of the transitions for the native BPTI spectrum (-239.2×10^{-42} cgsu/tyrosine). We may have overestimated tyrosine contributions to the native BPTI spectrum using curve resolution (see Discussion).

A second set included the two resolved negative bands centered at 241.5 and 231.7 nm and the positive one at 222.2 nm. We suggest that the positive band reflects the loss of the L_a transition and that either or both of the negative bands are the transitions due to the ionized tyrosines, as outlined under Discussion. The rotational strength of the 222.2-nm band is about the magnitude expected for tyrosine L_a transitions (Holladay & Puett, 1976); however, both the sign of the band and its relatively low wavelength are unusual.

[2] This value may be slightly underestimated, as perhaps 10% of the tyrosine-23 residues ($pK = 11.6$) are un-ionized at pH 12.5 (Snyder et al., 1976).

The negative band centered at 207.6 nm and the positive ellipticity at lower wavelengths were tentatively assigned to the ionized tyrosine and tyrosine B_b transitions.

Discussion

Comparison of the Native and Nativelike Spectra: Relevance to the Unfolding–Refolding Pathway. The CD spectra of native BPTI and refolded BPTI are not identical; therefore, the conformations of these two three-disulfide-bond inhibitors cannot be identical. All previous studies, including diagonal mapping, chromatographic and electrophoretic mobility, inhibitor activity, immunochemical reactivity, and UV difference spectroscopy (Creighton, 1978; Creighton et al., 1978; Kosen et al., 1980), had shown no dissimilarities in refolded BPTI and native BPTI. It is now apparent that these methods were not sufficiently sensitive to discriminate between small conformational differences. Similar differences were detected between the two disulfide species N^{SCM}_{SCM} and (30–51, 5–55).

The CD spectrum of refolded BPTI is about 6% less intense at almost all wavelengths than that of native BPTI. Because the decreased ellipticity occurs throughout the measurable wavelength region, it is difficult to assign a single or limited number of structural elements as the cause of the spectral changes. The near-UV portions of the two spectra differ; therefore, one or more of the tyrosines and/or disulfides must be in altered environments after refolding. Curve resolution is suggestive of the former as there are larger changes in the assigned tyrosine transitions than in those of the disulfides upon refolding. A similar argument can be made for the spectral changes found for N^{SCM}_{SCM} and (30–51, 5–55).

Heating solutions of refolded BPTI at temperatures below that required for measurable denaturation, followed by cooling, converted the CD spectrum of refolded BPTI to one indistinguishable from that of native BPTI. Refolded BPTI might then be trapped in a metastable conformation which reverts to a more nativelike state when sufficient conformational flexibility is permitted.

There is now additional evidence that the conformations of native and refolded BPTI are not identical. Recent NMR studies by States et al. (1980) have shown refolded BPTI to consist of a mixture of two populations, one indistinguishable from native BPTI, the other with a small difference in conformation. The latter was also observed to be converted to the former by gentle heating. Two tyrosines were found in altered environments.

The CD difference spectra of native BPTI minus N^{SCM}_{SCM} and of refolded BPTI minus (30–51, 5–55), both shown in Figure 4, are nearly identical; the structural alterations that exist between the pairs, native BPTI and N^{SCM}_{SCM}, and refolded BPTI and (30–51, 5–55), are therefore presumably also the same. Thus, the conformational differences that have been found between native BPTI and refolded BPTI are most likely not incorporated into the protein during the period of refolding that involves formation of the 14–38 disulfide but must have occurred prior to or at the formation of (30–51, 5–55).

While it is not known what features of refolding are responsible for the "incorrect" refolding of (30–51, 5–55) and refolded BPTI, the presence of incorrect cis-trans isomers of the peptide bonds adjacent to proline residues has been suggested by R. L. Baldwin to be one possible explanation. In general, each proline residue has one energetically favorable isomer in the folded state, but the cis and trans forms have comparable energies in the unfolded state. Unfolded protein molecules then equilibrate to a mixture in which each proline peptide bond has approximately 0.8 probability of being trans (Garthwohl & Wüthrich, 1976; Ramachandran & Mitra,

1976; Cheng & Bovey, 1977; Deslauriers et al., 1979). Isomerization is a relatively slow process, with a half-time of about 1 min at room temperature, and so is often rate limiting in protein folding (Brandts et al., 1975, 1977; Lin & Brandts, 1978). The kinetic importance in protein folding of this phenomenon has been demonstrated in the case of ribonuclease A (Garel & Baldwin, 1973, 1975; Schmid & Baldwin, 1978; Cook et al., 1979). BPTI has four proline residues at positions 2, 8, 9, and 13, all of which are in the trans conformation in the native state (Deisenhofer & Steigemann, 1975). With a probability of 0.8 of each residue being trans, and assuming each residue to be independent, only 41% of the molecules of unfolded, reduced BPTI would be expected to have all four proline residues in the trans conformation and thus be able to refold readily. Isomerization of the remaining 59% of the molecules could be rate limiting, but only 15–20% of the molecules have been observed to form incorrect disulfide bonds (Creighton, 1977b). The remainder of the molecules with one or more incorrect cis–trans isomers might have refolded to a nativelike conformation, with three correct disulfide bonds but incorrect proline isomers. The protein folded conformation might then greatly decrease the rate of isomerization, unless the temperature was increased substantially. There is evidence that ribonuclease, with the four disulfides intact, can refold to a nativelike conformation with one or more incorrect cis–trans isomers (Cook et al., 1979), and a number of other proteins show similar folding kinetics (Creighton, 1980b).

CD Spectrum of R: Relevance to the Unfolding–Refolding Pathway. It is important to show that the fully reduced inhibitor is a very disordered polymer without a limited number of preferred conformations which might direct the refolding pathway (Creighton, 1978). The fully reduced inhibitor, with its thiol groups carboxymethylated or carboxyamidomethylated, has been shown to be the least ordered and the least globular form of BPTI by its slowest migration during gel electrophoresis, which is not altered by a urea gradient (Creighton, 1979), by its limited solubility in certain buffer systems, by the equivalent reactivity against alkylating reagents of the six thiol groups, by the nearly complete solvent accessibilities of the aromatic amino acids, by its inability to bind antibodies raised from native BPTI, and by the inability of native BPTI to bind antibodies raised from the fully reduced and carboxymethylated protein (Creighton, 1975c; Creighton et al., 1978; Kosen et al., 1980). Further evidence has been provided in this paper against the possibility that R maintains stable conformational regions which direct the refolding process. The CD spectrum of R is that of a denatured protein (Fasman et al., 1970). Neither α helices nor β sheets appear to be present in significant quantities. There is no evidence for or against transitory secondary structures which might direct early refolding events (Karplus & Weaver, 1976).

The Gdn·HCl and temperature titration curves are not sigmoidally shaped, which would indicate the cooperative denaturation of a stable conformation, but rather are straight lines. The effect of low concentrations of Gdn·HCl cannot be significant, as the folding pathway is not altered substantially in the presence of 0.5 M Gdn·HCl (Creighton, 1981). The spectral change observed there is probably due to the increase in ionic strength of the solvent. Linear titration curves have been recorded in the cases of glucagon (Panijpan & Gratzer, 1974) and corticotropin (Holladay & Puett, 1976). The linear change indicates that a protein chain is flexible and converts through a series of conformations as solution conditions change.

Spectral Changes Due to the Reduction of the 14–38 Disulfide. In the next three sections, spectral features are correlated with known structural elements of native BPTI and N^{SCM}_{SCM}.

Three major spectral changes occur upon reduction of the 14–38 disulfide and are reflected in the difference spectrum of Figure 4. The loss of ellipticity between 265 and 320 nm is primarily like that expected of a disulfide; an estimation of the rotational strength ($\sim -2 \times 10^{-40}$ cgsu) is similar to that found for L-cystine and other disulfide models in solution (Kahn, 1972; Strickland, 1974; Holladay et al., 1976). A second change is the loss of the positive shoulder near 250–260 nm, and may be the loss of a second 14–38 disulfide transition. The disulfide is the only chromophore with potentially significant transitions in this region, and often, in model disulfide spectra, the two highest wavelength transitions are oppositely signed (Kahn, 1972). The third change, reflected in the 215–240-nm region of Figure 4, is believed due to changes in the peptide backbone, rather than in the aromatic residues. Small environmental changes are known to occur for tyrosines-10 and -35 and phenylalanine-33 as well as for the peptide region between residues 35 and 40 (Brunner et al., 1974; Snyder et al., 1976; Wagner et al., 1979a,b). Assignment of the differential band to changes of the L_a transitions of the aromatic residues appears unlikely for two reasons. The rotational strength of the differential band between 215 and 240 nm is approximately 3.7×10^{-39} cgsu, 3 times that of the L_a transition for glycyl-L-tyrosylglycine (Holladay & Puett, 1976). The magnitude of the spectral change would imply large conformational changes in the three aromatic amino acids, which is not the case. Solvent changes have also not been shown to significantly alter the magnitude of L_a transitions. Therefore, by default, peptide bond conformational changes are the most likely cause of the ellipticity changes in the 215–240-nm region.

Two small minima at 280 and 287.5 nm are also present in the difference spectra of Figure 4. These minima, assigned to changes in tyrosine transitions, indicate that there is some small effect on the tyrosines after reduction, which agrees with other observations (Snyder et al., 1976; Wagner et al., 1979a,b; Kosen et al., 1980). The curve resolutions indicated larger changes in the tyrosine transitions after reduction of the 14–38 disulfide, but that is probably incorrect. In order to completely fit the spectrum of N^{SCM}_{SCM}, it was necessary to also increase the disulfide transition. Such an increase is unlikely since the 30–51 and 5–55 disulfides are in regions of the protein which are not affected by reduction of the 14–38 disulfide (Snyder et al., 1976). The pH difference spectrum (Figure 10) indicated that the rotational strengths of the tyrosine transitions for native BPTI were nearly the same as those resolved for N^{SCM}_{SCM}; both of these were about 14–20% smaller than those resolved in the native BPTI spectrum.

The discrepancies in the near-UV between the curve resolutions and the difference spectra are easily explained, if the curve resolutions lacked the positive band centered near 260 nm, due to a possible 14–38 disulfide transition. This curve was not included in the resolution because there was the implicit assumption that a minimum number of transitions should completely fit a spectrum. In summary, the contributions by tyrosine transitions have probably been overestimated and those of disulfides underestimated in the curve resolution of the native BPTI (and the refolded BPTI) spectra.

Implications for Conformational Flexibility. The magnitudes of the tyrosine transitions in the spectra of the folded species are at least 5 times more intense than those of gly-

cyl-L-tyrosylglycine (Holladay & Puett, 1976). The greater intensities signify that, on the average, the four tyrosines have fewer conformational degrees of freedom than does the model tyrosine (Strickland et al., 1970, 1972; Holladay & Puett, 1976). This agrees with previous observations; tyrosines-23 and -35, and to a lesser extent tyrosine-21, are buried in the interior of the native protein which may restrict their conformational mobility (Huber et al., 1971; Deisenhofer & Steigemann, 1975). For example, tyrosine-35 is so immobilized by surrounding atoms that its NMR spectrum shows four ring proton resonances instead of two (Snyder et al., 1975; Wagner et al., 1979b).

The disulfide transitions may also reflect conformational flexibility and rigidity. The rotational strengths per disulfide of the transition assigned to the 30–51 and 5–55 disulfides in the spectrum of N^{SCM}_{SCM} or (30–51, 5–55) are nearly -1.0×10^{-39} cgsu. Disulfide transitions of this magnitude have been identified in the spectra of model disulfides when there is a known loss of conformational freedom due to either crystallization or a decrease in temperature (Kahn, 1972; Takagi & Ito, 1972). Thus, the 30–51 and 5–55 disulfides, which are buried in the protein, give rise to appreciable ellipticity, which is probably due to inherent asymmetry caused by conformational restrictions.

In contrast, the rotational strength associated with the 14–38 disulfide ($\sim -2.0 \times 10^{-40}$ cgsu, Figure 4) is much smaller, of a magnitude similar to that found for small flexible disulfides in solution. It is also similar to that found in the spectra of human growth hormone, bovine growth hormone, and ovine pituitary prolactin for certain disulfide transitions. Those disulfides presumably also lie on the surface, for they also can be selectively reduced in the absence of denaturants without the loss of overall protein conformation (Bewley, 1977). A similar rotational strength was found for the 6–20 disulfide of epidermal growth factor *after* disruption of the conformation by cyanogen bromide cleavage at methionine-21 (Holladay et al., 1976). We suggest that, unlike the 30–51 and 5–55 disulfides, the 14–38 disulfide does not have primarily a single conformation but has a greater degree of flexibility which results in the smaller rotational strength. It is on the surface of the protein and not constrained on all sides by other atoms. Theoretical considerations indicated that the region of the protein containing disulfide 14–38 is more flexible than other areas (M. Karplus, unpublished experiments). Therefore, it seems possible that the flexibility of this part of the protein includes the 14–38 disulfide, which consequently is considerably more flexible than either 30–51 or 5–55; the flexibility is reflected in the smaller magnitude of the disulfide transition.

Comparison of the CD Spectra of Native BPTI at Neutral and at Alkaline pH. The pH CD difference spectrum (Figure 10) was originally calculated to clarify the nature of the tyrosine and disulfide contributions to the spectrum of native BPTI; it has been useful in this regard, but there were a number of unexpected observations. The secondary structure of BPTI is intact at pH 12.5, whereas the four tyrosine residues are almost completely ionized (Sherman & Kassell, 1968; Vincent et al., 1971; Karplus et al., 1973; Wagner & Würthrich, 1975; Snyder et al., 1975), so that observed spectral changes should be due only to the ionization of tyrosines; this expectation was fulfilled. The pattern of the differential bands observed in Figure 10 does not resemble that expected if portions of the secondary structure were destroyed. The phenylalanine and disulfide transitions are not expected to be altered by pH changes. However, tyrosine transitions are known to shift to longer wavelengths as the hydroxyl groups are ionized. Further, at either neutral or alkaline pH, at least three transitions might be expected, including the B_b and L_a transitions in the far-UV (Strickland, 1974; Holladay et al., 1976; Ascoli et al., 1977).

The differential bands assigned to tyrosine and ionized tyrosine transitions are unusual in three respects. First, the L_a and B_b tyrosine transitions at neutral pH are predicted to be negative. To our knowledge, only the CD spectra of *cyclo*-L-alanyl-L-tyrosine and *cyclo*-L-tyrosyl-L-tyrosine have been shown to have negative far-UV tyrosine transitions (Snow et al., 1977). However, Woody (1978) had predicted a rotational strength for the L_a tyrosine transitions in the native BPTI spectrum as -1.215×10^{-39} cgsu/tyrosine after considering the interactions of the tyrosine side chains with the nearest-neighbor amide groups; this value is not much different in magnitude from the experimental one (Table V). Second, the position of the proposed L_a transition (222.2 nm) is at a shorter wavelength by 4–7 nm than that normally observed, but there is precedence for this in the L_a transition of the *cyclo*-L-alanyl-L-tyrosine CD spectrum. Third, the stronger of the two negative bands, which we assigned to the ionized tyrosine L_a transitions, is also at a shorter wavelength (231.7 nm rather than 240 nm) than that normally found. Although the other band is at a more typical wavelength, its intensity is much less than that expected if comparison to model compound spectra is made.

Comparison of Figures 2 and 9 indicates that the presumed L_a and B_b transitions effectively alter the shapes of the CD curves. The contributions of these transitions to the CD spectrum of native BPTI may at least partially explain why the linear least-squares analysis of the native BPTI spectrum failed to calculate the known amounts of secondary structures.

Acknowledgments

We thank Bayer AG for the generous gift of the inhibitor; L. A. Holladay, D. Puett, and R. G. Hammonds, Jr., of Vanderbilt University, Nashville, TN, for their hospitality, the introduction to the technique of curve resolution, and the use of the computer program for the calculations of secondary structure; J. Brunelle for the preparation of the computer programs used in the final resolutions of the CD spectra reported within; and A. Gal/at for helpful suggestions.

Appendix

Circular dichroism is a sensitive measure of protein conformation. Unfortunately, much of the information can be lost, as protein CD spectra are complicated by the presence of the electronic transitions of many chromophores in different environments. Curve resolution is a qualitative attempt to simplify a spectrum by isolating a minimum number of Gaussian curves which give a satisfactory fit and which relate, by their band parameters, to transitions resolved either in model compound spectra or by theoretical methods.

BPTI has three types of amino acids which absorb in the near-UV and potentially contribute to the CD spectrum: four tyrosines, three disulfides, and four phenylalanines. The spectra of nine different states of BPTI have been measured [five in this paper, including the pH 12.5 spectrum, and four by P. A. Kosen, T. E. Creighton, and E. R. Blout (unpublished experiments)]. Curve resolution has been used to qualitatively discern conformational differences between the tyrosines and disulfides of these species by comparison of the transitional rotational strengths; these are a measure of the relative flexibility or rigidity of the residues. No claim is made for the uniqueness of the resolutions, but they are internally self-consistent.

Experimentally, with the aid of a graphics display terminal, Gaussian curves were manually added, subtracted, and altered in position, intensity, and half-width until the sum of these bands visually agreed with an experimental spectrum. Then, a nonlinear least-squares fitting operation was performed by computer to minimize the difference between the experimental and theoretical spectra.

The choice of starting and final curves depended on the following limitations: (1) Only a minimum number of bands were included in a resolution. This is an obvious approximation: at least three disulfide transitions might be expected, yet in the spectra of this paper, only one is included and assumed to be an average. The same approximaton was made for the tyrosine transitions. (2) The placement and bandwidths of the tyrosine transitions were in line with those resolved in the spectra of glycyl-L-tyrosylglycine in aqueous solvents (Holladay & Puett, 1976). As the latter study included only the $0 + n(800)$ cm^{-1} vibronic series, we confined the resolutions to those transitions. Two features were examined before the final resolutions were accepted: (a) The separations of the tyrosine transitions were spaced as close as possible to 800 cm^{-1}. (b) The relative (but not absolute) intensities of the tyrosine transitions were similar to those in the glycyl-L-tyrosylglycine spectra. (3) Phenylalanine transitions are notoriously weak (Horwitz et al., 1969; Holladay et al., 1974) and in some spectra (P. A. Kosen, T. E. Creighton, and E. R. Blout, unpublished experiments) were omitted as an adequate fit was achieved without their inclusion. In the cases of native BPTI and the nativelike spectra, one phenylalanine transition was included, which is an obvious underestimation of phenylalanine contributions. The two shoulders near 260 and 265 nm are pH insensitive, a characteristic of phenylalanine transitions. As noted in Table III, the band assigned to the $0 + 3(800)$ cm^{-1} tyrosine transition could also contain a phenylalanine contribution, but an additional band was not included due to limitation 1. (4) Resolution of disulfide transitions was based on the work of Kahn (1972). These transitions are broad, are devoid of fine structure, and vary greatly in position, all of which makes their resolution difficult. Two factors determined the placement of disulfide transitions: (a) All ellipticity above 290 nm is due to disulfide transitions and must so be accounted. (b) When the ellipticity near 250–260 nm was intense relative to the near-UV minimum, as was the case in most spectra, the former was assumed due to disulfide transitions. Neither tyrosines and phenylalanines nor peptide bonds should be responsible for ellipticity near 250–260 nm, whereas disulfides do absorb there.

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Conformation of Gramicidin A in Phospholipid Vesicles: Circular Dichroism Studies of Effects of Ion Binding, Chemical Modification, and Lipid Structure†

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ABSTRACT: The effects of cation binding, chemical modification, and lipid structure on the conformation of the channel-forming polypeptide gramicidin A in phospholipid vesicles have been investigated by circular dichroism spectroscopy in order to aid in elucidating the mechanism of action of this antibiotic and to ascertain features necessary for maintenance of its structure in membranes. Gramicidin A is capable of adopting a number of different conformations, depending on its environment. In organic solvents, it forms a family of double-helical dimers, whereas in membranes, it forms an N-terminal to N-terminal dimer of single helices. None of the structures in a variety of hydrophilic and amphipathic organic solvents are equivalent to its structure in membranes. A mechanism for cation binding in which the channel widens (and consequently the helix foreshortens) had been proposed on the basis of studies of crystals formed from organic solvents. This study of gramicidin demonstrates that in membranes the helical pitch (and, therefore, width and length) of the molecule remains unaltered upon binding of ions and suggests that the ion channel binding mechanism must involve only small, local changes such as position of side chains or hydrogen bonds near the cation binding site. The effect of the membrane lipid thickness and organization on peptide conformation has been examined by altering fatty acid chain lengths and the lipid phase state, respectively. In addition, variations of peptide–lipid ratios indicate that peptide–lipid interactions must be important in maintaining the helical conformation. Conformational studies of chemically modified gramicidin molecules have been correlated with their conductance properties.

Gramicidin A[1] is a linear polypeptide antibiotic consisting of 15 hydrophobic amino acids of alternating L and D configuration, whose primary structure has been determined (Sarges & Witkop, 1965). It forms ion channels in membranes that are specific for small monovalent cations (Hladky & Haydon, 1972). Conductance and fluorescence measurements have demonstrated that the conducting species in membranes is the dimer (Veatch & Stryer, 1977; Bamberg & Lauger, 1973). The detailed structure of that dimer has been under investigation for a number of years. Urry first proposed that it consisted of two end-to-end single helices with a β-sheetlike hydrogen-bonding pattern, which he denoted π (L,D) (Urry, 1971). That the N-terminal to N-terminal dimer was the major form adopted in black lipid films was strongly suggested by conductance measurements using modified gramicidins (Bamberg et al., 1977; Bradley et al., 1978; Szabo & Urry, 1979). A minor component consisting of C-terminal to C-terminal dimers was later also considered to be a possibility

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[1] Abbreviations used: CD, circular dichroism; IR, infrared spectroscopy; NMR, nuclear magnetic resonance spectroscopy; DMPC, dimyristoylphosphatidylcholine; gramicidin A, a mixture of 85% gramicidin A, 10% gramicidin B, and 5% gramicidin C.

0006-2960/81/0420-5754$01.25/0