Folding of the Twisted β-Sheet in Bovine Pancreatic Trypsin Inhibitor†

Kuo-Chen Chou, George Némethy, Marcia S. Pottle, and Harold A. Scheraga*
Baker Laboratory of Chemistry, Cornell University, Ithaca, New York 14853-1301
Received June 21, 1985

ABSTRACT: The dominant role of local interactions has been demonstrated for the formation of the strongly twisted antiparallel β-sheet structure consisting of residues 18–35 in bovine pancreatic trypsin inhibitor. Conformational energy minimization has indicated that this β-sheet has a strong twist even in the absence of the rest of the protein molecule. The twist is maintained essentially unchanged when energy minimization is carried out by starting from the native conformation. By starting from a nontwisted β-sheet conformation of residues 18–35, a strongly twisted structure (higher in energy than the native) is obtained. The high twist of the native-like β-sheet is a consequence of its amino acid sequence, but it is enhanced strongly by interchain interactions that operate *within* the β-sheet. The existence of the twisted β-sheet structure does not require the presence of a disulfide bond between residue 14 and residue 38. It actually may facilitate the formation of this bond. Therefore, it is likely that the β-sheet structure forms during an earlier stage of folding than the formation of this disulfide bond. This study provides an example of the manner in which conformational energy calculations can be used to provide information about the probable pathway of the folding of a protein.

All β-sheets in globular proteins are right-twisted (Chothia, 1973; Salemme & Weatherford, 1981a,b; Richardson, 1981). The uniquely preferred direction and magnitude of the twist have been explained in terms of intra- and interchain interactions (Chou & Scheraga, 1982; Chou et al., 1982, 1983a,b). These interactions depend on the amino acid composition and sequence (Chou et al., 1983b). When a strongly twisted β-sheet is observed in a globular protein, it is important nevertheless to determine whether the extent of the twist is an intrinsic property of the amino acid sequence of the β-sheet, taken by itself, or whether it is affected strongly by interactions of the β-sheet with the rest of the protein molecule. An answer to this question is also important for revealing details of the folding process during which the native structure is formed. If the twisted β-sheet can exist (without untwisting spontaneously) in the absence of the rest of the molecule, it is possible that it forms during the early stages of the folding and may even help to direct the folding of other parts of the molecule.

In order to answer such questions for a specific protein, we have used conformational energy computations to study the conformational behavior of residues 18–35 of bovine pancreatic trypsin inhibitor[1] (Figure 1). These residues appear in a twisted hairpin-like structure, consisting of a two-strand antiparallel β-sheet formed by residues 18–24 and 29–35 and of a turn that connects the two strands, formed by residues 25–28. This β-sheet is unusually highly twisted: the sheet has a total twist of about 180° (Deisenhofer & Steigemann, 1975), i.e., 53° per two residues. This value can be compared with the overall observed range of 0–60° per two residues for β-sheets in many globular proteins (Richardson, 1981) and with computed twists of up to 32° per two residues for various homopoly(amino acid)s (Chou et al., 1983b).

The twisting of this β-sheet may play a role in the folding of BPTI and in the formation of the disulfide bond between the half-cystine residues 14 and 38 that are located near the ends of the hairpin-like structure. This disulfide bond forms last during the oxidative refolding of reduced BPTI (Creighton & Goldenberg, 1984). An intermediate form that has two disulfide bonds but lacks the disulfide bond between residue 14 and residue 38 has been shown to adopt a conformation very close to that of the native protein (Kosen et al., 1980, 1981, 1983). One of the objectives of the present study is to determine whether the twisted hairpin-like antiparallel structure formed by residues 18–35 can exist by itself or whether it needs the rest of the molecule to form or to prevent it from untwisting or unfolding.

We have minimized the energy of hairpin-like antiparallel β-sheet structures formed by residues 18–35 in the absence of the rest of the BPTI molecule, starting from twisted and flat (nontwisted) conformations, respectively, in order to determine whether structures with a strong twist can be obtained from both starting points. Additional computations were carried out on a similar β-sheet that is formed by two *separate* polypeptide strands, in order to determine whether the presence of the turn that connects the two strands in the hairpin-like structure influences the twist significantly.

Methods

Conformational energies were computed as a function of all backbone and side-chain dihedral angles, with the standard amino acid geometry (bond lengths and bond angles) and the energy parameters of the ECEPP algorithm in its updated version ECEPP/2 (Momany et al., 1975; Némethy et al., 1983). The energy was obtained as the sum of electrostatic, nonbonded, hydrogen-bond, and torsional energies. Energy minimizations were carried out either with the MINOP function optimizer algorithm (Dennis & Mei, 1975), with a convergence limit of 0.0001 kcal/mol, or with the SUMSL general unconstrained optimizing algorithm (Gay, 1983). Computations were performed on a Prime 550 minicomputer with an attached Floating Point Systems AP-120B array processor (Pottle

† This work was supported by research grants from the National Institute of General Medical Sciences (GM-14312) and the National Institute on Aging (AG-00322) of the National Institutes of Health, U.S. Public Health Service, and from the National Science Foundation (DMB84-01811). It was also supported in part by the National Foundation for Cancer Research.

[1] Abbreviations: BPTI, bovine pancreatic trypsin inhibitor; rms, root mean square.

0006-2960/85/0424-7948$01.50/0

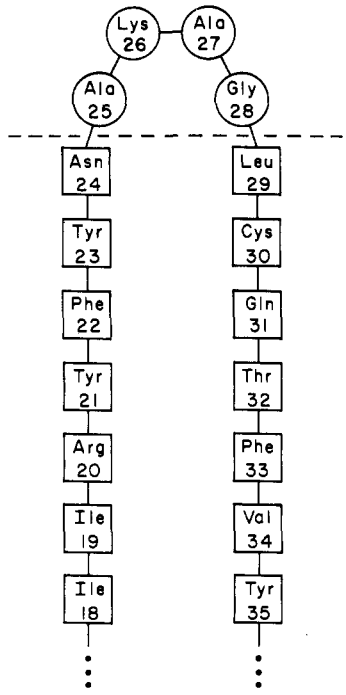

FIGURE 1: Amino acid sequence of residues 18–35 of BPTI. Residues indicated by squares form the two strands of the antiparallel β-sheet. Residues indicated by circles form the turn connecting the two strands of the β-sheet.

et al., 1980). The standard conventions for the nomenclature of peptide conformations are followed (IUPAC–IUB Commission on Biochemical Nomenclature, 1970).

Selection of Starting Conformations. (a) In the computations on the hairpin-like structure, the amino acid sequence of residues 18–35 of BPTI was used, with *N*-acetyl and *N'*-methylamide end groups. Energy minimization with respect to all dihedral angles was carried out from two starting conformations. (i) The first is the native conformation in BPTI. The starting backbone dihedral angles (columns 2–4 of Table I) were those derived by fitting the X-ray coordinates (Deisenhofer & Steigemann, 1975) with standard ECEPP residue geometry (Swenson et al., 1978; Dunfield & Scheraga, 1980). (ii) The second is a flat hairpin structure. Residues 18–24 and 29–35 were assigned dihedral angles $(\phi, \psi, \omega) = (-130°, 126°, 180°)$ and a relative position and orientation (see next paragraph) that correspond to a flat (nontwisted) β-sheet with optimal interchain hydrogen bonds, by a geometrical fitting procedure (Chou et al., 1982). The conformation of the connecting link, consisting of residues 25–28, was assigned by means of a fitting procedure that allows smooth joining of polypeptide fragments with standard residue geometry (K.-C. Chou, G. Némethy, M. S. Pottle, and H. A. Scheraga, unpublished results).

(b) In the computations for two separate polypeptide chains that form an antiparallel β-sheet, the two strands had amino acid sequences corresponding to residues 18–24 and 29–35 of BPTI, respectively. Both chains were terminated with *N*-acetyl and *N'*-methylamide end groups. Energy minimization was carried out from two sets of starting conformations, using not only all dihedral angles as variables but also the external variables, i.e., the Euler angles and translational displacements that describe the relative position and orientation of the two chains (Chou et al., 1982). (i) The first conformation was the native-like (twisted) structure. The dihedral angles and the relative position and orientation of the strands (as described by the external variables) were identical with those of the native starting conformation of the hairpin structure, as described in (a) (i) above. (ii) The second set of conformations consisted of flat β-sheet structures. Several sets of backbone dihedral angles that correspond to flat (nontwisted) β-sheet structures were chosen as starting points [Figure 4 of Chou et al. (1983b)]. In each starting conformation, the chains were juxtaposed to form optimal, i.e., near-linear, hydrogen bonds between the backbone N–H and C=O groups, by a geometrical fitting procedure (Chou et al., 1982). Within this set, the lowest energy structure was obtained when minimization was started from $(\phi, \psi, \omega) = (-130°, 126°, 180°)$ for every residue. Only this structure will be described under Results. Minima obtained from the other starting points had higher energies and are not considered further.

(c) One energy minimization each was carried out for the isolated *individual* extended strands, consisting of residues 18–24 and of residues 29–35, respectively, with *N*-acetyl and *N'*-methylamide end groups. This computation was performed to ascertain the extent of twisting of each peptide chain in the absence of interstrand interactions. The starting dihedral angles were $(\phi, \psi, \omega) = (-130°, 126°, 180°)$ for every residue.

Conformations of the Side Chains. The starting side-chain dihedral angles used in every minimization were those of the ECEPP-fitted native structure (Swenson et al., 1978; Dunfield & Scheraga, 1980). It was not necessary to investigate other side-chain conformations because the purpose of this study was not an exhaustive search of various conformations but the assessment of constraints resulting from various extents of twist of the backbone.

Definition of the Twist. The twist of the two strands of the β-sheet is described here in terms of δ, the twist of the individual strands. δ has been defined [Figure 1 of Chou et al. (1982)] as the dihedral angle formed by the atoms O_i–C'_i–C'_{i+2}–O_{i+2} of residues i and $i + 2$ along the chain. It is a function of the helical parameter n, i.e., the number of residues per turn of a helical structure, which in turn depends on the backbone dihedral angles. The average twist $\langle\delta\rangle$ of a polypeptide chain has been defined (Chou & Scheraga, 1982; Chou et al., 1982) as

$$\langle\delta\rangle = (360°/r)\sum_{i=1}^{r}(2 - |n_i|)/n_i$$

where r is the number of residues of the chain and n_i is the helical parameter, computed for each residue i. For a flat (nontwisted) β-sheet, $n = \pm 2$ and $\langle\delta\rangle = 0$. Right-twisted β-sheets are characterized by $n < -2$ and $\langle\delta\rangle > 0$.

RESULTS

The backbone dihedral angles of the ECEPP-fitted native conformation (Dunfield & Scheraga, 1980) and of the computed minimum-energy structures are listed in Table I. The energy and the average twist of each structure are summarized in Table II.

There is very little change of the conformation upon minimization starting from the native structure. The mean change in the backbone dihedral angles is only 4°, and the average twist remains nearly constant: $\langle\delta\rangle$ changes from 53.1 to 54.6°. Structure 1 is thus very close to the native structure (Figure 2A). Its energy is 40 kcal/mol lower than that of an extended conformation. The results indicate that the highly twisted native-like hairpin structure formed by residues 18–35 is a relatively stable structure; i.e., it is in a local energy minimum that does not necessarily unfold even in the absence of the rest of the BPTI molecule.

Structure 2, shown in Figure 2B and obtained by minimization starting from the nontwisted hairpin structure (Figure 2C) with $\langle\delta\rangle = 0$, also exhibits a strong right-handed twist,

Table I: Dihedral Angles (deg) of Residues 18–35 of BPTI in the Refined Native Structure and of the Computed Minimum-Energy Structures

minimized structure				1			2			3			4		
				single hairpin structure minimized from						two separate peptide chains minimized from					
	native structure[a]			twisted (native) structure			flat structure			twisted structure			flat structure		
residue	ϕ	ψ	ω	ϕ	ψ	ω	ϕ	ψ	ω	ϕ	ψ	ω	ϕ	ψ	ω
Ile-18	−111	121	180	−108	125	180	−86	88	178	−111	123	175	−81	127	179
Ile-19	−80	113	180	−80	109	−178	−86	130	−168	−77	112	180	−91	98	−175
Arg-20	−120	173	180	−119	168	−177	−84	137	173	−117	172	−177	−74	153	174
Tyr-21	−116	135	180	−117	144	174	−137	132	−176	−115	136	180	−135	136	−175
Phe-22	−138	134	180	−138	144	−177	−164	166	174	−138	136	−179	−161	162	−178
Tyr-23	−73	143	180	−74	138	176	−97	147	170	−72	141	−179	−156	160	172
Asn-24	−118	102	180	−116	110	−175	−162	137	−173	−118	102	179	−151	146	−177
Ala-25	−54	−30	180	−64	−35	−173	−161	168	173						
Lys-26	−69	−69	180	−68	−62	−179	62	−92	175						
Ala-27	−53	−40	180	−60	−37	177	−152	36	178						
Gly-28	98	10	180	95	16	−177	−84	−143	177						
Leu-29	−142	−179	180	−145	175	180	−151	169	177	−142	180	180	−157	167	175
Cys-30	−97	144	180	−93	146	180	−67	104	178	−95	142	−179	−150	153	−176
Gln-31	−132	168	180	−132	163	−176	−77	82	−178	−129	167	−177	−139	164	−174
Thr-32	−91	144	180	−91	149	172	−83	118	172	−88	142	178	−79	84	169
Phe-33	−155	141	180	−154	148	−178	−150	160	−170	−158	143	180	−146	155	−176
Val-34	−66	128	180	−77	128	176	−71	107	177	−69	127	180	−92	134	178
Tyr-35	−111	132	180	−114	134	180	−160	150	178	−112	132	180	−157	153	179

[a] Swenson et al., 1978; Dunfield & Scheraga, 1980.

Table II: Average Twist and Conformational Energy of the β-Sheet Formed by Residues 18–35 of BPTI in the Refined Native Structure and in the Computed Minimum-Energy Structures

minimized structure		1	2	3	4		
		single hairpin structure minimized from		two peptide chains minimized from		isolated extended chains minimized from	
	native structure[a] (single hairpin)	twisted (native) structure	nontwisted structure	twisted structure	nontwisted structure	nontwisted structure	
residues	18–35	18–35	18–35	18–24 and 29–35	18–24 and 29–35	18–24	29–35
average twist $\langle\delta\rangle$ (deg)							
first strand	43.3	48.9	35.0	46.0	34.1	36.8	
second strand	63.0	60.2	35.3	61.0	27.3		24.9
two-stranded β-sheet	53.1	54.6	35.2	53.5	30.5	NA[b]	NA
energy (kcal/mol)							
single hairpin structure							
total energy[c]		−87.5[d]	−75.6	NA	NA	NA	NA
two-chain structure							
total energy[c]		NA	NA	−70.1	−66.6	NA	NA
intrastrand energy							
first strand		NA	NA	−12.6	−41.1	−42.5	
second strand		NA	NA	−1.4	−16.5		−16.6
interstrand energy		NA	NA	−56.1	−9.1	NA	NA

[a] Before minimization. [b] NA, not applicable. [c] See footnote 2 of the text. [d] Minimization of residues 18–35 from an extended conformation gives a total energy of −47.6 kcal/mol.

with $\langle\delta\rangle = 35.2°$, although this twist is about 18° smaller than that in the native structure. The strong twist obtained upon minimization indicates that there is a strong intrinsic tendency for right-handed twisting in the amino acid sequence of residues 18–35. Upon minimization, a local minimum-energy conformation with a moderate degree of twist has been reached. This is indicated by the higher value of the energy of structure 2, as compared with that of structure 1.

Closely similar results were obtained when the energy was minimized for two separate chains forming a β-sheet. When the native-like, twisted two-strand structure was used as starting point, the twist remained nearly unchanged, viz., $\langle\delta\rangle = 53.5°$, and the dihedral angles of the resultant minimum-energy structure 3 were nearly the same as those of structure 1. In structure 4, obtained by starting from two chains in a nontwisted arrangement, $\langle\delta\rangle = 30.5°$. This value is only $\approx 5°$ less than the twist of the analogous structure 2, even though some individual dihedral angles differ considerably in the two structures. The energy of the more highly twisted structure 3 is lower than that of structure 4, paralleling the relative energies of structures 1 and 2, respectively.[2]

The twist and the intrastrand energy of the individual strands in structure 4 are closely similar to those computed for the two isolated strands in their minimum-energy conformation (as seen from the last three columns of Table II). This result indicates that structure 4, as well as the related structure 2, represents local conformational energy minima, with properties determined largely by the intrinsic tendencies of the sequences of the two strands. Therefore, the enhanced twisting and lower energy of structures 1 and 3 arise from interstrand interactions.

[2] The energies of only structures 1 and 2 can be compared with each other. Those of structures 3 and 4 also can be compared with each other. A comparison of the energies listed for structures 1 or 2 with those of structures 3 or 4 is meaningless, however, because these energies refer to polypeptides with different amino acid composition (Momany et al., 1975).

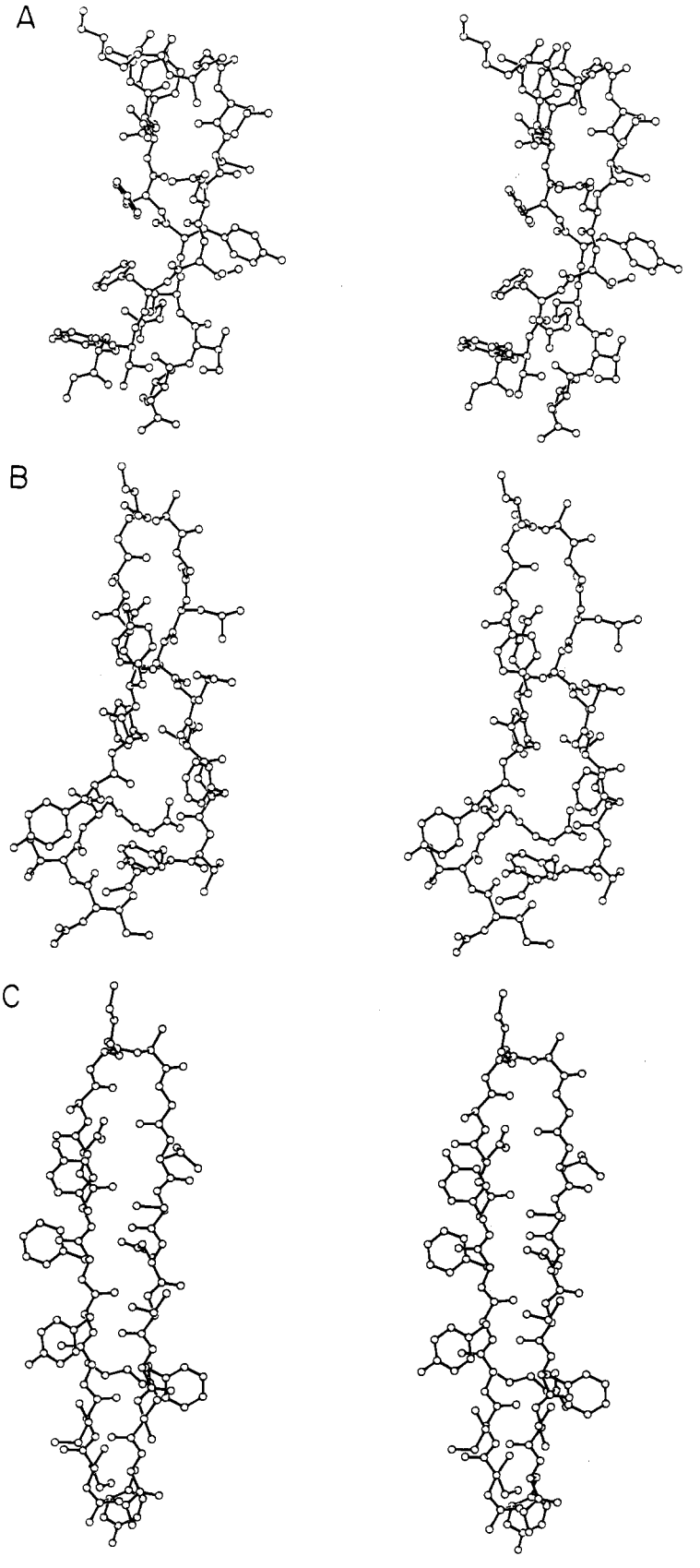

FIGURE 2: Stereoscopic drawings of hairpin structures formed by residues 18–35 of BPTI. (A) Strongly twisted β-sheet structure 1 obtained by minimization starting from the native conformation. (B) Moderately twisted structure 2 obtained by minimization starting from a flat (nontwisted) β-sheet conformation. (C) Flat (nontwisted) β-sheet structure, used as the starting point from which structure 2 has been obtained. The direction of viewing is such that the bend at the top is seen in a similar orientation in all three drawings, viz., residue Lys-26 appears to the left of residue Ala-27 (cf. Figure 1). The difference in the extent of twisting between (A) and (B) is seen clearly. Twisting is fairly uniform in (A), but increases from top to bottom in (B). The individual strands and the two-strand structure in (C) are not twisted even though some of the hydrogen bonds near the bottom are not linear. Their small deviation from linearity is caused by limited convergence in the numerical minimization procedure used to join the connecting link to the β-strands. This does not influence the energy-minimization procedure that results in the right-twisted structure (Chou et al., 1983a).

The close similarity of the native β-sheet and of that in structures 1 and 3 is indicated by the small root mean square deviation between them. On the basis of all atoms of residues 18–24 and 29–35, the rms deviations between the native structure and structures 1 and 3 are 0.59 and 0.46 Å, respectively, and that between structures 1 and 3 is 0.52 Å. In contrast, the rms deviation between structures 1 and 2, having different twists (cf. Figure 2A,B), is 4.59 Å, while that between structures 3 and 4 is 8.23 Å.

Discussion

The results show clearly that the amino acid sequences of residues 18–24 and 29–35 favor the existence of a strong right-handed twist. The isolated extended chains take up twisted minimum-energy conformations with $\langle\delta\rangle = 36.8°$ and 24.9°, respectively (columns 7 and 8 of Table II). Packing of the two strands into the *lowest-energy* twisted conformation considerably enhances their twisting, to $\langle\delta\rangle = 46.0°$ and 61.0°, respectively (structure 3). Thus, the high twist results from the combined effect of the amino acid sequences themselves and of packing of two strands in the β-sheet. On the other hand, structure 4, obtained when the two extended flat chains are *first* packed and then allowed to twist, represents a local conformational energy minimum, prevented by steric hindrance from twisting into the more stable packing arrangement.

The interchain energy between the two strands of the β-sheet favors structure 3 relative to structure 4, as shown in Table II. Interchain interactions in the more stable structure 3 greatly enhance the twist over that preferred for the isolated strands. Similar effects of interchain interactions have been observed previously (Chou et al., 1983a).

While the results presented here cannot be taken to imply that the twisted β-sheet is the most stable conformation for residues 14–38, this β-sheet is shown to be a favorable structure, as compared with an unfolded conformation.

The results obtained with two separate polypeptide chains and those with one chain folded into a hairpin-like structure are closely similar to each other, as seen from a comparison of structures 1 and 2 with structures 3 and 4, respectively. The presence of residues 25–28, connecting the two strands of the hairpin-like β-sheet, increases the twist in both structures 1 and 2, although its effect is minor as compared with the effect of packing of the two extended chains.

It can be concluded (i) that the amino acid sequences of residues 18–24 and 28–35 of BPTI have a strong intrinsic tendency for right-handed twisting of the β-sheet, (ii) that packing of these two sequences enhances the twist to the value observed in native BPTI, and therefore (iii) that the rest of the BPTI molecule is not necessary to cause this twisting or to stabilize the twisted conformation. While the connecting loop, consisting of residues 25–28 is important during the formation of the hairpin-like structure, its presence is not required for the existence of the high right-handed twist.

Several deductions can be made from these results regarding some details of the folding process in the formation of the native three-dimensional structure of BPTI. First, it is less likely that a *nontwisted* antiparallel hairpin-like structure, formed by residues 18–35, would be on the pathway of folding, because subsequent twisting of such a structure results in an intermediate energy conformation like structure 2, with much less twist than the native-like structure with lowest energy (structure 1). An energy barrier would have to be overcome in the conversion of structure 2 into 1. While it cannot be excluded that such a conversion might be facilitated by interactions with the rest of the BPTI molecule, the conversion is likely to require a rearrangement that involves at least a partial disruption of the hairpin structure 2. Second, it is therefore more probable that the folding of residues 18–35 would proceed by the initial formation of a short hairpin-like bend structure (residues 25–28), followed by zipper-like growth of the hydrogen-bonded antiparallel β-sheet. In such a folding process, each residue can adopt the extended conformation with the proper dihedral angles for the highly twisted structure without experiencing steric constraints. Third, it is possible to generate a hairpin structure with a high twist, without the rest of the molecule necessarily being in a native-like conformation. The present study does not exclude possible favorable interactions with the rest of the molecule during the folding process, but it shows that such interactions are not necessary for maintaining the twisted conformation of residues 18–35 that is observed in the native protein.

Additional deductions can be reached regarding the disulfide bond between residue 14 and residue 38. The relative stability of structure 1 indicates that it can form before the disulfide bond is made; i.e., the disulfide bond is not necessary to stabilize the antiparallel β-sheet. On the other hand, when this β-sheet has been formed, it constrains cysteine residues 14 and 38 to be near each other, thus facilitating the formation of the disulfide bond. This disulfide bond forms in a late stage of renaturation of BPTI (Creighton & Goldenberg, 1984). Presumably, the antiparallel β-sheet is formed at an earlier stage of folding. Furthermore, the threading of the polypeptide chain through a covalent loop in the native structure of BPTI can be explained readily if it is assumed that the β-sheet twists before the last of the three disulfide bonds is formed (Kikuchi et al., 1985).

Conclusions

We have shown that the strongly twisted antiparallel β-sheet structure formed by residues 18–35 of BPTI can exist in a local energy minimum without untwisting or unfolding even in the absence of the rest of the protein molecule. A strong right-handed twist is an intrinsic property of the amino acid sequence of residues 18–24 and 29–35, although it is necessary to pack the two fragments into a double-stranded antiparallel β-sheet in order to obtain the very high observed twist of 53° per two residues. The twisted structure can exist in the absence of a disulfide bond between residue 14 and residue 38. It may even facilitate the formation of this bond. Therefore, it is likely that the β-sheet structure forms in a stage of folding of BPTI that precedes the formation of this disulfide bond.

Acknowledgments

We thank Dr. P. W. Mui and M. Vásquez for helpful discussions and S. Rumsey for help with computer programming.

Registry No. BPTI, 9087-70-1.

References

Chothia, C. (1973) *J. Mol. Biol. 75*, 295–302.

Chou, K.-C., & Scheraga, H. A. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 7047–7051.

Chou, K.-C., Pottle, M., Némethy, G., Ueda, Y., & Scheraga, H. A. (1982) *J. Mol. Biol. 162*, 89–112.

Chou, K.-C., Némethy, G., & Scheraga, H. A. (1983a) *J. Mol. Biol. 168*, 389–407.

Chou, K.-C., Némethy, G., & Scheraga, H. A. (1983b) *Biochemistry 22*, 6213–6221.

Creighton, T. E., & Goldenberg, D. P. (1984) *J. Mol. Biol. 179*, 497–526.

Deisenhofer, J., & Steigemann, W. (1975) *Acta Crystallogr., Sect. B: Struct. Crystallogr. Cryst. Chem. B31*, 238–250.
Dennis, J. E., & Mei, H. H. W. (1975) Technical Report 75-246, Department of Computer Sciences, Cornell University, Ithaca, NY.
Dunfield, L. G., & Scheraga, H. A. (1980) *Macromolecules 13*, 1415–1428.
Gay, D. M. (1983) *ACM Trans. Math. Software 9*, 503–524.
IUPAC–IUB Commission on Biochemical Nomenclature (1970) *Biochemistry 9*, 3471–3479.
Kikuchi, T., Némethy, G., & Scheraga, H. A. (1985) *J. Comput. Chem.* (in press).
Kosen, P. A., Creighton, T. E., & Blout, E. R. (1980) *Biochemistry 19*, 4936–4944.
Kosen, P. A., Creighton, T. E., & Blout, E. R. (1981) *Biochemistry 20*, 5744–5754.
Kosen, P. A., Creighton, T. E., & Blout, E. R. (1983) *Biochemistry 22*, 2433–2440.
Momany, F. A., McGuire, R. F., Burgess, A. W., & Scheraga, H. A. (1975) *J. Phys. Chem. 79*, 2361–2381.
Némethy, G., Pottle, M. S., & Scheraga, H. A. (1983) *J. Phys. Chem. 87*, 1883–1887.
Pottle, C., Pottle, M. S., Tuttle, R. W., Kinch, R. J., & Scheraga, H. A. (1980) *J. Comput. Chem. 1*, 46–58.
Richardson, J. S. (1981) *Adv. Protein Chem. 34*, 167–339.
Salemme, F. R., & Weatherford, D. W. (1981a) *J. Mol. Biol. 146*, 101–117.
Salemme, F. R., & Weatherford, D. W. (1981b) *J. Mol. Biol. 146*, 119–141.
Swenson, M. K., Burgess, A. W., & Scheraga, H. A. (1978) in *Frontiers in Physicochemical Biology* (Pullman, B., Ed.) pp 115–142, Academic Press, New York.

Isolation and Characterization of 101-β-Lysozyme That Possesses the β-Aspartyl Sequence at Aspartic Acid-101

Hidenori Yamada, Tadashi Ueda, Ryota Kuroki, Toshimitsu Fukumura, Takanori Yasukochi, Tatori Hirabayashi, Kahee Fujita, and Taiji Imoto*

Faculty of Pharmaceutical Sciences, Kyushu University 62, Maidashi, Higashi-ku, Fukuoka 812, Japan

Received June 14, 1985

ABSTRACT: In the reaction of the intramolecular cross-linking between Lys-13 (ε-NH_3^+) and Leu-129 (α-COO^-) in lysozyme using imidazole and 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide hydrochloride [Yamada, H., Kuroki, R., Hirata, M., & Imoto, T. (1983) *Biochemistry 22*, 4551–4556], it was found that two-thirds of the protein (both the recovered and cross-linked lysozymes) showed a lower affinity than the rest against chitin-coated Celite, an affinity adsorbent for lysozyme. The protein with the reduced affinity was separated on chitin-coated Celite affinity chromatography and found to be slightly different from native lysozyme in the elution position of the tryptic peptide of Ile-98–Arg-112 on reversed-phase high-performance liquid chromatography. In contrast with native lysozyme, the limited hydrolysis of this abnormal tryptic peptide of Ile-98–Arg-112 in 6 N HCl at 110 °C gave a considerable amount of β-aspartylglycine. Therefore, it was concluded that two-thirds of the protein obtained from this reaction possessed the β-aspartylglycyl sequence at Asp-101–Gly-102. As a result, we obtained four lysozymes from this reaction, the derivative with the β-aspartyl sequence at Asp-101 (101-β-lysozyme), the cross-linked derivative between Lys-13 and Leu-129 (CL-lysozyme), the CL-lysozyme derivative with the β-aspartyl sequence at Asp-101 (101-β-CL-lysozyme), and native lysozyme. In the ethyl esterification of Asp-52 in lysozyme with triethyloxonium fluoroborate [Parsons, S. M., Jao, L., Dahlquist, F. W., Borders, C. L., Jr., Groff, T., Racs, J., & Raftery, M. A. (1969) *Biochemistry 8*, 700–712; Parsons, S. M., & Raftery, M. A. (1969) *Biochemistry 8*, 4199–4205], the same bond rearrangement was detected in the same ratio. Therefore, it is concluded that the Asp-52 ethyl ester lysozyme reported had been a mixture of the derivatives with the α- and β-aspartyl sequences at Asp-101. The mechanism for the formation of 101-β-lysozyme in these reactions is discussed.

Recently, we have reported the preparation of the intramolecularly cross-linked lysozyme between the ε-amino group of Lys-13 and the α-carboxyl group of Leu-129 by the carbodiimide reaction catalyzed by imidazole (Yamada et al., 1983). During the investigation of the nature of the cross-linked lysozyme, we noticed that the thermal stability of the recovered lysozyme from this reaction was much reduced compared with native lysozyme. This observation suggested that some unknown lysozyme derivatives were formed in this reaction. Recently, we have prepared chitin-coated Celite as an affinity adsorbent for lysozyme and found the affinity chromatography on this resin to be sometimes very efficient for separation of the chemically modified lysozyme derivatives (Yamada et al., 1985). On utilizing this chromatography, we found the recovered lysozyme and the cross-linked lysozyme obtained previously were both mixtures of two components, respectively. In both cases, the components with lower affinities were the derivatives with the β-aspartyl sequence at Asp-101. Furthermore, Asp-52 ethyl ester lysozyme produced in the reaction of lysozyme with triethyloxonium fluoroborate (Parsons et al., 1969; Parsons & Raftery, 1969) was also found to be a mixture of two derivatives with the α- and β-aspartyl sequences at Asp-101.

Experimental Procedures

Materials. Five times recrystallized hen egg white lysozyme was donated from Eisai Co. (Tokyo, Japan). 1-Ethyl-3-[3-(dimethylamino)propyl]carbodiimide hydrochloride (EDC)[1]

0006-2960/85/0424-7953$01.50/0