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Unperturbed Dimensions of Disordered Proteins Containing an Interchain Disulfide Cross-Link

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ABSTRACT: Mean-square unperturbed radii of gyration, $\langle s^2 \rangle_0$, have been calculated for several proteins cross-linked via an interchain disulfide bond. Thirty different polypeptide chains were used. Characteristic ratios tend to be smaller for cross-linked proteins than for the uncross-linked chains, although exceptions to this generalization do exist. Random flight statistics tend to overestimate the value of g, defined as the ratio of $\langle s^2 \rangle_0$ for the cross-linked protein to $\langle s^2 \rangle_0$ for an analogous linear polypeptide chain containing the same number of amino acid residues. The parameter f_i, defined as the ratio of the $\langle s^2 \rangle_0$ for the ith uncross-linked polypeptide chain and the cross-linked protein, is usually more accurately estimated by random flight statistics than is g. When the cross-link connects two chains of identical amino acid sequence, the values of f_i obtained via random flight statistics are within 6% of those provided by rotational isomeric state theory.

The ordered structures adopted by proteins in their native states can be completely disrupted by suitable changes in solvent composition.[1] Concentrated guanidine hydrochloride containing a disulfide bond reducing agent, such as mercaptoethanol or dithiothreitol, is the most thoroughly characterized such system.[2-5] The reducing agent serves to rupture any disulfide cross-links present, thereby permitting characterization of the disordered polypeptides by the methods customarily applied to linear polymers. Presumably the ordered structures would also be disrupted if guanidine hydrochloride were used without a disulfide bond reducing agent, but the intact cross-links would bring about alterations in the dimensions of the molecules. Our present objective is to examine the conformational consequences arising from the presence of a single intact interchain disulfide cross-link in denatured proteins. Calculations have been performed using rotational isomeric state theory[6,7] in the form appropriate for branched molecules.[8,9]

Computations

The formulation of the configuration partition function, generator matrices, and expression for the mean-square unperturbed radius of gyration of the α-carbon atoms can be found in ref 10 and 11. All amino acid residues, except glycyl, L-prolyl, and those L-cysteinyl residues involved in cross-link formation, were treated as L-alanyl residues in the formulation of the generator matrices. The $\gamma 2$ transformation matrix from ref 12 was used for those L-prolyl residues followed by another L-prolyl residue. Polypeptide chains used, along with literature citations for the amino acid sequences and the number of amino acid residues, n, are collected in Table I. Cross-linked molecules contain one interchain disulfide bond. The disulfide bonds considered do not necessarily occur in the proteins in their native states.

Results and Discussion

Characteristic Ratios. The characteristic ratios for cross-linked and uncross-linked polypeptide chains will be defined as $\langle s^2 \rangle_0/(n-1)l_p^2$, where $\langle s^2 \rangle_0$ is the unperturbed mean-square radius of gyration of the n α-carbon atoms in the molecule. For uncross-linked polypeptide chains this definition is equivalent to $\langle s^2 \rangle_0/n_p l_p^2$, where n_p is the number of virtual bonds. The length of the virtual bond, l_p, is 3.8 Å.[13]

Figure 1 presents the characteristic ratios obtained for the molecules considered. Characteristic ratios for the uncross-linked polypeptide chains are denoted by squares. They range from a low of 0.81 (α-trypsin C chain) to a high of 1.41 (α-tropomyosin). All but three polypeptide chains with $n \geq 100$ have a characteristic ratio which lies between 1.00 and 1.29. The exceptions are cytochrome c, globin, and α-tropomyosin. The incorporation of a few glycyl or L-prolyl residues in a poly(L-alanine) chain reduces its characteristic ratio.[14,15] Therefore the high characteristic ratio for α-tropomyosin arises because only three glycyl residues, and no L-prolyl residues, occur out of a total of 284 amino acid residues. In contrast, 16–21% of the amino acid residues in cytochrome c and globin are glycyl or L-prolyl, leading to the unusually low characteristic ratios for these proteins. The average characteristic ratio for those polypeptide chains having $n \geq 100$ is 1.13, with a standard deviation of 0.11.

Characteristic ratios for the polypeptide chains cross-linked via a disulfide bond are also shown in Figure 1. Filled circles denote cases where both polypeptide chains have the same amino acid sequence. Multiple cross-linked forms are possible

Table I
Polypeptide Chains Studied

Polypeptide	Source	n	Site of Cys	Sequence[a]
Thrombin A chain	Bovine	49	22	D-109
α-Trypsin C chain	Bovine	98	12, 58, 85	D-105
β_2-Microglobulin	Human	100	25, 81	*b*
Calcium binding protein	Hake	108	18	S-84
Calcium binding protein	Carp 3	108	18	S-84
Cytochrome *c*	C. oncopelti	111	26	S-15
Coelomic hemerythrin	T. pyroides	113	50	S-23
Coelomic hemerythrin	P. gouldii	113	50	S-23
Myohemerythrin	T. pyroides	118	34, 99	*c*
α-Trypsin B chain	Bovine	123	7, 109, 116	D-105
Histone III (f_3)	Bovine	135	96, 110	*d*
Homoglobin α chain	Horse	141	104	D-59
Hemoglobin α chain	Gray kangaroo	141	104	D-62
Hemoglobin α chain	Rhesus monkey	141	104	*e*
Hemoglobin α chain	Human	141	104	D-56
Hemoglobin β chain	Rabbit	146	93	D-70
Hemoglobin γ chain	Human	146	93	D-77
Globin	Bloodworm	147	30	S-25
Myoglobin	Human	153	110	S-27
Coat protein	TMV HR	156	27	D-286
Endolysin	Bacteriophage λ	157	119	S-30
Coat protein	TMV U2	158	27	D-286
Coat protein	TMV dahlemense	158	27	D-285
Coat protein	TMV vulgare	158	27	D-285
Troponin C	Bovine	161	35, 84	*f*
κ-Casein	Bovine	169	11, 88	S-81
Thrombin β chain	Bovine	260	28, 44, 123, 177, 188, 202, 232	D-109
α-Tropomyosin	Rabbit	284	190	*g*
Carboxypeptidase A	Bovine	307	138, 161	D-126
Immunoglobulin γ1 chain	Human	446	226, 229	D-264

[a] "D" denotes the page in M. O. Dayhoff, "Atlas of Protein Sequence and Structure", Vol. 5, National Biomedical Research Foundation, Georgetown University Medical Center, Washington, D.C., 1972. "S" denotes the page in the 1973 supplement. [b] P. A. Peterson, B. A. Cunningham, I. Berggard, and G. E. Edelman, *Proc. Natl. Acad. Sci. U.S.A.*, **69**, 1697 (1972). [c] G. L. Klippenstein, J. L. Cote, and S. E. Ludlaw, *Biochemistry*, **15**, 1128 (1976). [d] R. J. Delange, J. A. Hopper, and E. L. Smith, *Proc. Natl. Acad. Sci. U.S.A.*, **69**, 882 (1972). [e] G. Masuda, T. Maita, H. Ota, and H. Takei, *J. Protein Res.*, **2**, 99 (1970). [f] J. P. van Eerd and K. Takahashi, *Biochemistry*, **15**, 1171 (1976). [g] A. D. McLachlan, M. Stewart, and L. B. Smillie, *J. Mol. Biol.*, **98**, 281 (1975).

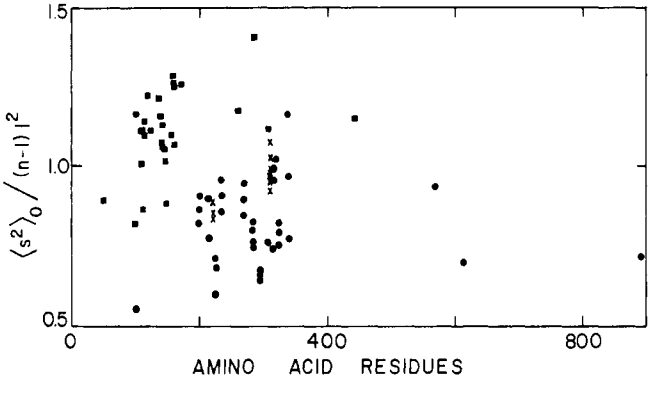

Figure 1. Characteristic ratios for cross-linked proteins (● and ×) and for the uncross-linked polypeptide chains which constitute these proteins (■). The proteins used are listed in Table I. Points for cross-linked proteins in which both polypeptide chains have the same amino acid sequence are denoted by circles. In some cases a single point represents two or more cross-linked proteins which give virtually identical results.

if the polypeptide chain contains more than one L-cysteinyl residue. For example, the polypeptide chain of κ-casein contains L-cysteinyl residues at positions 11 and 88. There are three possible ways of cross-linking two κ-casein chains via a single disulfide bond: 88–88, 11–88, and 11–11. The characteristic ratios are 0.77, 0.97, and 1.16, respectively (Table II). Each of these results is represented by a filled circle in Figure 1. In contrast, only one point is presented for carboxypeptidase and for the immunoglobulin γ1 chain because the characteristic ratios for the three cross-linked forms of these proteins are virtually identical. The results presented in Table II show that higher characteristic ratios are obtained as the L-cysteinyl residues involved in cross-link formation near an end of the polypeptide chain, as expected.[16,17] Mixed disulfides always yield the intermediate characteristic ratios.

Crosses represent cases where the disulfide bond unites polypeptide chains of different amino acid sequence. Trypsin is represented by three crosses, corresponding to the 13–143, 115–216, and 122–189 disulfide bonds of trypsinogen.[18] Seven crosses, one for each possible disulfide bond between the A and B chains, are presented for thrombin.

The characteristic ratios for the cross-linked polypeptides range from a low of 0.56 for the thrombin A chain to a high of 1.16 for one of the cross-linked forms of κ-casein. When n exceeds 100, the average characteristic ratio is 0.81, with a standard deviation of 0.13. The characteristic ratios of the cross-linked proteins tend to be lower than those obtained with the uncross-linked polypeptide chains, but the scatter is such that some overlap does occur.

With the exception of thrombin, the characteristic ratio of the cross-linked molecule is smaller than that of the uncross-linked constituent polypeptide chains. The A chain of thrombin, which is quite short, has a characteristic ratio of 0.89 when not cross-linked. Characteristic ratios of 0.93–1.07 are obtained for the molecules which result from cross-linking the A chain to the much larger B chain.

Table II
Conformational Properties for Various Kinds of Disulfide Bonds between Identical Polypeptide Chains

Protein	n	Disulfide	$\langle s^2\rangle_0/(n-1)l_p^2$	g	f_1	g/g_{rf}	$f_1/f_{1,rf}$
β_2-Microglobulin	100	25–25	0.82	0.65	0.70	0.90	1.02
		25–81	0.86	0.69	0.67	0.92	1.00
		81–81	0.91	0.72	0.64	0.94	0.98
Myohemerythrin	118	34–34	0.86	0.69	0.71	0.99	0.99
		34–99	0.91	0.73	0.67	0.98	1.01
		99–99	0.96	0.77	0.63	0.97	1.01
Histone III (f_3)	135	96–96	0.84	0.68	0.72	0.99	0.99
		96–110	0.89	0.72	0.68	0.99	0.99
		110–110	0.94	0.76	0.64	0.98	0.99
Troponin C	161	84–84	0.65	0.58	0.82	0.93	1.02
		35–84	0.72	0.65	0.74	0.94	1.02
		35–35	0.79	0.71	0.67	0.95	1.01
κ-Casein	169	88–88	0.77	0.58	0.82	0.93	1.02
		11–88	0.97	0.73	0.65	0.94	1.00
		11–11	1.16	0.87	0.54	0.95	0.99
Carboxypeptidase A	307	161–161	0.69	0.60	0.80	0.96	1.01
		138–161	0.70	0.61	0.80	0.97	1.01
		138–138	0.70	0.61	0.80	0.96	1.01
Immunoglobulin γ1 chain	446	226–226	0.72	0.62	0.80	0.99	1.00
		226–229	0.72	0.62	0.80	1.00	1.00
		229–229	0.72	0.63	0.79	1.00	0.99

One comparison between experimentally determined dimensions and those calculated via rotational isomeric state theory is possible. Light-scattering measurements have been conducted on denatured tropomyosin in 5 M guanidine hydrochloride.[19] The experimental results were $\langle s^2\rangle^{1/2} = 110$ Å, $M = 74\,000 \pm 5000$, $[\eta] = 0.45$ dL/g, and $A_2 = 7.13 \times 10^{-4}$ mol cm^3/g^2. Holtzer et al.[19] interpreted these results as an indication that the sample consisted of two cross-linked polypeptide chains. Their conclusion is supported by subsequent work,[20,21] which has shown that the sulfhydryl groups in α- and β-tropomyosin are readily oxidized by atmospheric oxygen, leading to the formation of an interchain disulfide bond. By using the experimentally determined molecular weight, second virial coefficient, and intrinsic viscosity in the Orofino–Flory equation,[22] Holtzer et al.[19] obtained an expansion factor of 1.44 and $\langle s^2\rangle_0^{1/2} = 76.4$ Å. We have recalculated the expansion factor, using the experimentally determined molecular weight, second virial coefficient, and radius of gyration in the appropriate form of the Orofino–Flory equation. Our result is an expansion factor of 1.32, which gives $\langle s^2\rangle_0^{1/2} = 83.3$ Å. The dimensions calculated for cross-linked α-tropomyosin via rotational isomeric state theory yield $\langle s^2\rangle_0^{1/2} = 87.5$ Å, a result which stands in excellent agreement with that deduced from the measurements of Holtzer et al.[19]

In order to determine the sensitivity of the computed dimensions to the assignment of statistical weights in the L-cystinyl residue,[10] computations for the thrombin A chain, β_2-microglobulin, histone f_3, myoglobin, α-tropomyosin, and the immunoglobulin γ1 chain were repeated with s_1–s_{16} all set equal to unity. In no case did the characteristic ratio change by as much as 0.001. The flexibility in the C^α–C^β–S–S–C^β–C^α segment is sufficient to allow this portion of the L-cystinyl residue to act virtually as a free joint so far as the unperturbed dimensions are concerned.

The unperturbed dimensions of disordered, uncross-linked proteins have been shown[15] to be somewhat sensitive to small changes in the relative energies of the two minima in the conformational energy map for an L-prolyl residue followed by a nonprolyl residue.[14] Computations for the thrombin A chain, β_2-microglobulin, histone f_3, myoglobin, and the immunoglobulin γ1 chain were repeated with the relative energies of these two minima altered by ±1 kcal/mol. The greatest change in characteristic ratio (11%) occurred with the cross-linked immunoglobulin γ1 chain, which contains 37 L-prolyl residues per polypeptide chain of 446 residues. Changes in the unperturbed dimensions for the other four cross-linked proteins averaged 4%. The effect of the use of different conformational energy maps for an L-prolyl residue followed by another L-prolyl residue was not explored because of the infrequent occurrence of the –Pro–Pro– sequence in the proteins studied.

The consequences of seemingly minor changes in amino acid sequence can be deduced from an examination of results calculated for four families of proteins: calcium binding protein, hemerythrin, hemoglobin α chain, and tobacco mosaic virus coat protein. The polypeptide chains in each family contain the same number of amino acid residues, and the position of the L-cysteinyl residue is constant. Results are summarized in Table III. The characteristic ratio for cross-linked hake calcium binding protein is substantially lower than that obtained from carp, which is consistent with the difference of occurrence of the glycyl residue in these proteins. The slight differences in the characteristic ratios for the cross-linked hemerythrins arise as a consequence of the change in position of one glycyl residue (position 9 in T. pyroides and 79 in P. gouldii). A lower characteristic ratio is obtained when this glycyl residue is closer to the middle of the polypeptide chain. The characteristic ratios of the hemoglobin α chains increase as the sum of the number of glycyl and L-prolyl residues decreases, but the reverse effect is seen with the tobacco mosaic virus coat proteins. An L-prolyl residue occurs at the amino terminus of TMV U2, but not dahlemense or vulgare. This L-prolyl residue will be ineffective in depressing the characteristic ratio.

The Parameter *g*. The ratio of the mean-square radii of gyration for branched and unbranched molecules having the same number of bonds is customarily denoted by g.[21,22] Ambiguity exists in the definition of the pertinent unbranched chain for a cross-linked protein, such as thrombin, which contains two different polypeptide chains. In such cases we define $\langle s^2\rangle_0$ for the unbranched molecule as the average of $\langle s^2\rangle_0$ for the sequences A chain–B chain and B chain–A chain. From the viewpoint of random flight statistics, the cross-linked polypeptide chains consist of four branches emanating

Table III
Summary of the Unperturbed Dimensions for Four Families of Cross-Linked Proteins[a]

Proteins	Source	Gly[b]	Pro[c]	$\langle s^2\rangle_0/(n-1)l_p^2$	g^d	f_1^e
Calcium binding protein	Hake	12	0	0.77	0.76	0.65
	Carp	8	0	0.90	0.78	0.61
Hemerythrin	T. pyroides	7	4	0.71	0.61	0.80
	P. gouldii	7	4	0.68	0.60	0.80
Hemoglobin α chain	Horse	10	6	0.74	0.64	0.71
	Gray kangaroo	11	5	0.76	0.67	0.70
	Rhesus monkey	9	6	0.80	0.66	0.70
	Human	7	7	0.82	0.68	0.70
Coat protein	TMV dahlemense	6	8	0.95	0.76	0.63
	TMV vulgare	6	8	0.99	0.78	0.63
	TMV U2	5	10	1.02	0.79	0.63

[a] The number of amino acid residues per polypeptide chain and the location of the disulfide cross-links are given in Table I. [b] Number of glycyl residues in one polypeptide chain. [c] Number of L-prolyl residues in one polypeptide chain. [d] Random flight statistics yield the following g: 0.80 (calcium binding protein), 0.63 (hemerythrin), 0.71 (hemoglobin α chain), and 0.79 (coat protein). [e] Random flight statistics yield the following f_1: 0.62 (calcium binding protein), 0.79 (hemerythrin), 0.70 (hemoglobin α chain), and 0.63 (coat protein).

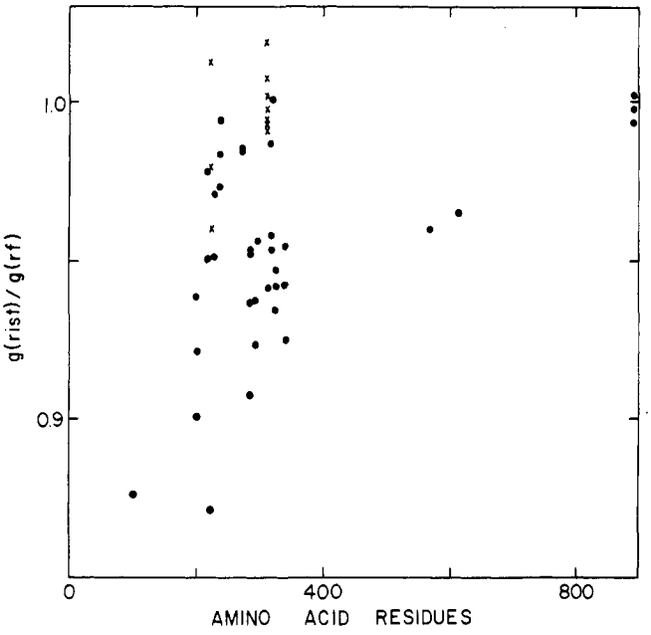

Figure 2. Ratio of the g calculated via rotational isomeric state theory to that obtained from random flight statistics for cross-linked proteins listed in Table I. The circles denote cases where both polypeptide chains have the same amino acid sequence.

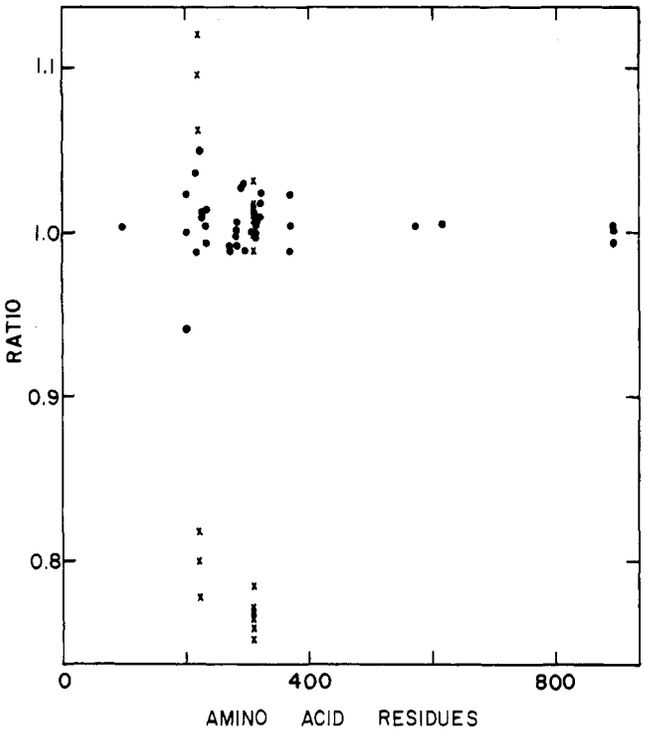

Figure 3. Ratio of the f_i computed via rotational isomeric state theory to that obtained with random flight statistics. The circles denote cross-linked proteins where both polypeptide chains are of the same amino acid sequence. Results are shown as a function of the number of amino acid residues in the cross-linked protein.

from a common point, the L-cystinyl residue. Random flight statistics yield

$$g = N^{-3}\sum(3Nn_j^2 - 2n_j^3) \quad (1)$$

where n_j is the number of virtual bonds in branch j and $N = \Sigma n_j$.[16,17] Results obtained from eq 1 will be denoted by g_{rf}, while g will signify values obtained using rotational isomeric state theory.

Table II presents g and g/g_{rf} for several proteins. The ratio g/g_{rf} is less sensitive than g or the characteristic ratio to changes in disulfide bonding, as is shown dramatically by the results reported for κ-casein. Table III demonstrates that g is less sensitive than the characteristic ratio to minor changes in amino acid sequence. Figure 2 presents g/g_{rf} for all of the cross-linked proteins. Only six entries exceed unity. The smallest values are obtained with the cross-linked cytochrome c (0.87) and cross-linked thrombin A chain (0.88). Deviations of g/g_{rf} from unity tend to decrease as the size of the protein increases. This observation, and the tendency for g/g_{rf} to lie below unity, are in accord with the correlations established between the size of the limiting characteristic ratio and the behavior of cross-linked polypeptides of finite molecular weight.[11]

The parameter f_1. The previous paper[11] introduced the term f_i, defined as the ratio of the mean-square radii of gyration for the ith polypeptide chain and the cross-linked protein. Calculated results are presented in Tables II and III. The f_i share with g the characteristic of being less sensitive than the unperturbed dimensions to minor changes in amino acid sequence, as shown by Table III. They are sensitive to

changes in cross-linking pattern, as demonstrated by κ-casein.

The ratios of the f_i calculated by matrix methods to those calculated by random flight statistics cluster much closer to unity than do the corresponding ratios involving g, provided attention is confined to cross-linked proteins in which both polypeptide chains are of the same amino acid sequence. This conclusion is apparent from Figures 2 and 3 or from comparison of the last two columns of Table II. A similar statement cannot be made if the cross-linked protein consists of polypeptide chains which differ in amino acid sequence. For example, the ratio of $\langle s^2 \rangle_0$ for the A chain of thrombin to $\langle s^2 \rangle_0$ for this chain cross-linked to the B chain lies between 0.75 and 0.78. We conclude that f_1 for cross-linked proteins is likely to be well approximated (to within about 6%) by eq 2[11] provided both polypeptide chains are of identical amino acid sequence.

$$f_1 = (n_1 + n_2)N^2[\sum(3Nn_j^2 - 2n_j^3)]^{-1} \quad (2)$$

Branches 1 and 2 constitute polypeptide chain 1.

The square root of f_i gives the ratio of a linear dimension of the ith polypeptide chain and the cross-linked polypeptide. This information can be used to assess the effect of the reduction of an interchain disulfide bond on the gel chromatography of disordered proteins,[23] provided the expansion factors are comparable for both molecules. For those cases where the cross-linked polypeptide chains are identical in amino acid sequence, $f_i^{1/2}$ ranges from 0.73 to 0.90. Consequently the cross-linked molecule will pass through the gel permeation column more quickly than the uncross-linked molecules, which is in accord with expectation.

Thrombin presents an interesting case in which f_i may exceed unity. The $f_i^{1/2}$ for the A chain are 0.36–0.39, depending upon the mode of cross-linking to the B chain. In contrast, the $f_i^{1/2}$ for the B chain are 0.96–1.03, indicating that cross-linked thrombin and the individual B chains will virtually coellute.

Acknowledgment. Supported in part by National Science Foundation Grant BMS 72-02416 A01 and in part by a fellowship from the John Simon Guggenheim Memorial Foundation.

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Structural Properties of Double-Stranded Helical Poly(γ-benzyl D-L-glutamate) in Solution. Comparison with Some Solution Properties of Linear Gramicidin

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Received October 28, 1976

ABSTRACT: The double-stranded helical conformations of alternating PBD-LG found in the solid state have been studied by infrared, circular dichroism, and NMR techniques in solution in methylene chloride, chloroform, dioxane, and collidine. The infrared and CD properties of the solutions, transconformations between single- and double-stranded helices and transconformations within the family of double helices, support the hypothesis that the conformation in solution is the same as that found after evaporation of the solvent, namely the $\pi\pi_{DL}^{7.2}$, $\pi\pi_{DL}^{9.0}$, and $\pi\pi_{DL}^{10.8}$ helices, depending on the solvent. An attempt to identify the conformations of linear gramicidin is made on the basis of the CD spectra and the infrared frequencies conformation relationship established for PBD-LG. However, owing to the great number of different conformations observed for the antibiotic, no firm conclusion can yet be drawn, except for the probable existence of the double antiparallel helical structure.

Two types of conformations have been proposed for gramicidin A, a channel forming natural alternating D-L-peptide;[1] these are a family of π_{DL} helices[2-5] and a family of double stranded helical conformations.[6,7] However, these structures were only postulated on the basis of spectroscopic results. They have not yet been firmly established owing to a lack of crystallographic data. In order to test the possible existence of these conformations, strictly alternating poly(γ-benzyl D-L-glutamate) (PBD-LG)[8] has been examined and its structure studied by fibre crytallography. In the solid state, this synthetic poly(D-L-peptide) has now been shown to exist in several helical conformations, among which are the α_{DL} helix, the $\pi_{DL}^{4.4}$ helix,[9,10] a member of the π_{DL} family, and four double-stranded helical conformations: the $\pi\pi_{DL}^{5.6}$, $\pi\pi_{DL}^{7.2}$,