Synthesis and Properties of High Molecular Weight Polypeptides Containing Proline[1)]

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High-purity *N*-carboxy-L-proline anhydride (*N*-carboxy-2-pyrrolidinecarboxylic acid anhydride) was synthesized. We used triethylamine instead of expensive Ag_2O to remove HCl during the synthesis. Copolypeptides with a random sequence of L-proline (Pro) with glycine, L-alanine (Ala), L-α-aminobuthyric acid (Abu), L-Norvaline (Nva) or L-leucine (Leu) were synthesized by copolymerization of the corresponding *N*-carboxy-α-amino acid anhydrides in solution. Copolypeptides of Pro with Ala or Abu were partially soluble in water. However, the copolypeptides of proline with Nva or Leu with longer side chains were insoluble in water. The conformation of water-soluble copolymer at various pH was analyzed by circular dichroism (CD). The structures of the polypeptides in aqueous solution were almost independent of the pH, and were in a collagen-like conformation.

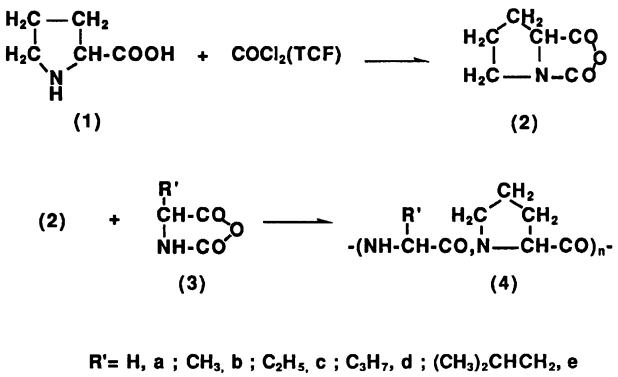
Polypeptides have been synthesized by the polymerization of *N*-carboxy-α-amino acid anhydrides (NCAs) or active esters of amino acids. The preparations of NCAs, their reactions and their polymers have been studied in some detail.[2)] Since high-molecular-weight polymers can be easily made from NCAs, and show a sharp molecular-weight distribution, many homopolypeptides and copolypeptides could be successfully synthesized using NCAs. However, these high-molecular-weight polypeptides are mostly insoluble in water but soluble in acidic organic solvents, such as trifluoroacetic acid, dichloroacetic acid or hexafluoroisopropanol. Therefore, physicochemical studies concerning these polypeptides have been undertaken in organic solvents or the solid state.[3,4)] Water-soluble polypeptides will be very useful as models of proteins and for drug-delivery systems. Although water-soluble synthetic polypeptides with charged side chains have been synthesized, their structures depend on the solvent pH. For some applications, water-soluble polypeptides whose structures are independent of the pH are desirable. Poly-DL-alanine is partly soluble in water. We previously reported that copolypeptides containing DL-tryptophan and alanine are partly soluble in water.[5)] Water-soluble polypeptides without any charged side chains or D-amino acids are little known, except for poly-L-proline (poly(Pro)). We have suggested that those polypeptides which have few inter-molecular hydrogen bonds or which do not take an ordered conformation as an α-helix or a β-sheet will be soluble in water.

In order to synthesize water-soluble copolypeptides whose conformation is invariant of the solvent pH, we synthesized copolypeptides using NCA of Pro, which tends to break the hydrogen bonds and standard secondary structures. We found that copolypeptides with Ala or Abu were partially soluble in water. We also succeeded in the synthesis of high-purity Pro NCA using triethylamine to remove the HCl generated during the synthesis. Previously, expensive Ag_2O was used for this purpose.

Experimental

Synthesis of Proline NCA(2) (Scheme 1). Pro (10 g, 0.087 mol) (**1**) was suspended by stirring in dry tetrahydrofuran (THF, 400 ml) in a three-necked, 1-liter round-bottom flask. Dry phosgene made from trichloromethyl chloroformate (TCF) was bubbled into the suspension at 40°C until a clear solution was obtained. The solvent and excess phosgene were removed in vacuo at room temperature, leaving NCA. Hexane (100 ml) was added onto the oily precipitate, and then allowed to stand at −20°C overnight. Hexane was removed by decantation. The oily residue was dried in order to remove any residual hexane under reduced pressure, and dissolved in acetonitrile (100 ml). Then, triethylamine (0.03 mol) was slowly added by stirring with the acetonitrile solution at −20°C. After being kept at −20°C overnight, the solvent was removed in vacuo, to obtain an oily product. The oily product was soluble in diisopropyl ether (300ml). The solvent was removed under reduced pressure to obtain a 60 ml solution; one volume of hexane was added. After 24 h at −20°C, the crystalline product was collected by filtration, purified by multiple recrystallization and dried in vacuo at room temperature; yield 3 g. 24.4%, mp 40°C.[8)] Found C, 50.77; H, 5.01; N, 9.61%. Calcd for $C_6H_7O_3N$: C, 51.50; H, 4.96; N, 9.93%.

Another NCA was prepared in high purity and good yield by a previously reported method.[9)]



Scheme 1. Synthesis of Pro NCA and polypeptides containing Pro.

Table 1. Synthesis and Properties of Copolypeptides Containing Proline

No.	NCA ratio (Pro/X)		Conversion Concn/mol dm^{-3}	(%)	Water soluble fraction(A) (%)[a]	(Pro/X)[b]	η_{sp}/c[c]	Water insoluble fraction (B) (%)	(Pro/X)	η_{sp}/c
1.	Pro		0.26	60;ACN Insol.	100	100	0.34[d]			
2.	Pro/Gly	;33/66	0.26	64;ACN Insol.	98	25/75	0.18[e]			
3.	Pro/Gly	;50/50	0.27	50;ACN Insol.	98	30/70	0.18[e]			
4.	Pro/Ala	;25/75	0.20	98;DCE Insol.	52	31/69	0.35[d]	43	13/87	0.24[e]
5.	Pro/Ala	;50/50	0.20	82;ACN Insol.	79	43/57	0.24[d]			
6.	Pro/Abu	;15/85	0.30	70;ACN Sol.	48	17/83	0.15[d]			
7A.	Pro/Abu	;50/50	0.30	50;ACN Sol.	40	46/54	0.12[d]			
7B.				38;ACN Insol.	82	81/19	0.03[d]			
8.	Pro/Nva	;66/34	0.20	50;ACN Insol.	0			100	56/44	0.31[e]
9.	Pro/Leu	;25/75	0.20	98;DCE Sol.	0			100	16/84	1.16[e]

a) Percentage of water-soluble fraction. b) After dialysis with a membrane of molecular-weight cut-off 3000. c) Before dialysis. d) c=0.25 g/100 cm^3 in dichloroacetic acid. e) c=0.5 g/100 cm^3 in trifluoroacetic acid.

Polymerization of NCAs. NCAs were copolymerized in acetonitrile or 1,2-dichloroethane under the conditions given in Table 1.

Preparation of Copoly(Pro, Abu) (Scheme 1). Pro NCA (0.93 g, 6.6 mmol) (**2**) and Abu NCA (**2c**, 0.85 g, 6.6 mmol) (**3b**) were dissolved in acetonitrile or 1,2-dichloroethane (45 ml). Butylamine (4.8 mg, 0.066 mmol) as an initiator was then added, and allowed to stand at 30°C for 21 d. After the reaction, the precipitated copolymer was separated on a sintered-glass filter, washed with acetonitrile, and dried in vacuo at room temperature. A four-tenth gram of the copolymer was obtained. Other copolypeptides were prepared by an analogous method.

Copolypeptides Soluble in Water. The copolypeptide (1.0 g) was placed in distilled water (100 ml), and kept for 24 h at 20—25°C. An insoluble polymer was separated on a sintered-glass filter, and then washed with water. The residue was dried in vacuo at room temperature. The filtrate was lyophilized to give a water-soluble copolypeptide.

Viscosity Measurement. The viscosities of the copolypeptides were measured in a solution of dichloroacetic acid or trifluoroacetic acid using an Ostwald viscometer at 25°C.

Amino Acid Composition of Copolypeptides. The amino acid compositions of water-soluble copolypeptides were determined from the NMR spectra of aqueous solutions of copolypeptides (2 mg ml^{-1}, D_2O): δ=3.60 and 3.80 (Pro CH_2), 1.35 (Ala CH_3), 0.97 (Abu CH_3). The amino acid compositions of water-insoluble copolypeptides were also determined with trifluoroacetic acid solutions (2 mg ml^{-1}): δ=0.91 (Nva CH_3) and 0.91 (Leu $(CH_3)_2$).

Gel Filtration. High-performance liquid chromatography was performed on a Shimadzu LC6A under a previously reported condition.[5]

Spectroscopic Measurement. The CD and optical rotatory disperssion (ORD) spectra were recorded on a JASCO J-20A, as previously reported.[5]

Results and Discussion

Solubility. Pro NCA was copolymerized with Gly NCA, Ala NCA, Nva NCA or Leu NCA in solution to produce copolypeptides. The polymerization conditions and properties of the copolypeptides are summarized in Table 1. Although acetonitrile is known to be a poor solvent of polypeptides, poly(Pro, Abu) was partly soluble in acetonitrile. Poly(Pro) was highly soluble in water, as expected; poly(Pro, Gly), poly(Pro, Ala), and poly(Pro, Abu) were partly soluble, but poly(Pro, Nva) and poly(Pro, Leu) were insoluble. In a series of copolypeptides containing DL-tryptophan, copoly(DL-Trp, Ala) was partly soluble in water; copoly(DL-Trp, Abu) was insoluble.[5]

Molecular Weight. The existing formulas that correlate the degree of polymerization of the copolypeptide and viscosity of the peptide solution do not always hold for the polypeptides prepared in this study. Gel permeation chromatography was then carried out in order to correlate the specific viscosity and molecular weight of the copolymers. The estimated weight-average molecular weight for polymer No. 1 with a reduced specific viscosity of 0.39 in dichloroacetic acid was found to be 30000. This suggests that these copolypeptides have a chain length that is sufficiently long to take some secondary structure.

CD Spectra. The reduced mean residue ellipticity [θ'] values are given in Table 2. The CD spectra of poly(Pro) and collagen were studied.[10] The ellipticity bands between 185 and 235 nm are used to discriminate the poly(Pro) type-I conformation (right-handed helix with *cis* peptide bonds) from the poly(Pro) type-II conformation, as found in collagen (left-handed helix with *trans* peptide bonds). A negative strong band at 199 nm, a negative weak one at 232 nm and a positive strong one at 215 nm were used to assign the poly(Pro) type-I conformation, while a positive strong peak near to 206 nm was used to assign the poly(Pro) type-II conformation. Water-soluble poly(Pro) and poly(Pro, Gly) exhibited a peak minimum at 206 nm in water and a 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP) solution. For all three polymers (No. 1—3), the CD spectra indicate that the proline type-II conformation prevails over a

Table 2. Conformation of Copolypeptides Containing Proline Shown in Table 1

No.	Amino acid composition	Solvent[a)]	CD Peak nm	$[\Theta']$[b)]	Conformation
1.	Pro	H_2O, pH4.4	206	−30000	Collagen
		HFIP	206	−13300	Collagen
2.	Pro/Gly ;25/75	H_2O, pH4.3	202—204	−8000	
		HFIP	204	−11200	
3.	Pro/Gly ;30/70	H_2O, pH4.4	206	−10300	Collagen
		H_2O, pH2.1	206	−7400	Collagen
		H_2O, pH6.9	206	−8800	Collagen
		H_2O, pH8.5	206	−5200	Collagen
		HFIP	203	−8700	
4.	Pro/Ala ;31/69	H_2O, pH1.8	200	−18000	
		H_2O, pH6.9	202	−14800	
		H_2O, pH8.5	200	−16200	
5.	Pro/Ala ;43/57	H_2O, pH1.8	206	−19100	Collagen
		H_2O, pH7.0	204	−24600	
		H_2O, pH11.0	206	−18800	Collagen
		HFIP	204	−5500	
6.	Pro/Abu ;17/83	H_2O, pH3.5	200	−7000	
		H_2O, pH7.0	200	−6300	
		H_2O, pH9.0	200	−6700	
		HFIP	202	−16000	
7A	Pro/Abu ;46/54	H_2O, pH3.5	202	−13000	
		H_2O, pH7.0	204—206	−11100	
		H_2O, pH9.0	206	−13100	Collagen
7B	Pro/Abu ;81/19	H_2O, pH4.0	216	−2700	
			226—228	+800	
		H_2O, pH7.0	214	−5100	
			228	+1000	
		H_2O, pH8.5	212	−19800	
			228—230	+1900	
8.	Pro/Nva ;56/44	HFIP	202	−9000	
9.	Pro/Leu ;16/84	(HFIP Insoll. Benzene Sol. b_0[c)] = −170)			

a) pH control was done by NaH_2PO_4, HCl and NaOH. b) The reduced mean residue ellipiticity. c) Moffit parameter of optical rotatory dispersion.

wide pH range.

Poly(Pro, Ala) (No. 4) and Poly(Pro, Abu) (No.6) have a negative peak at 200 nm. Fasman reported a strong negative CD peak at 197 nm and a positive peak at 217 nm for random coil poly(Lys) and poly(Lys, Leu) in aqueous solution.[11] Doty reported a strong negative peak at 202 nm and a weak positive peak between 210 and 235 nm for disordered poly(Glu) in aqueous solution.[12,13] Polymer No. 4 has a negative peak at 200 nm, whose intensity is less than that of random coil poly(Glu) (202 nm $[\theta']=50000$), but no positive peak between 210 and 235 nm. These patterns may well result from a spectral summation of the poly(Pro) type-II conformation and a disordered conformation. Poly(Pro, Ala) (No.5) and poly(Pro, Abu) (No.7) have a negative peak at 206 nm, which corresponds to the poly(Pro) II conformation. In polymer No.7, the negative peak shifted to 200 nm upon lowering the solution pH. Except for the poly(Pro. Abu) (No.7), the pH dependence of CD was negligible. Polymers No.4 and No.6 have a lower proline content and smaller absolute $[\theta']$ values than do polymers No.5 and No.7, respectively. These results indicate that the fraction of the poly(Pro) type-II conformation increases with increasing proline content. The acetonitrile-insoluble and water-soluble fraction of polymer No.7B has a weak negative peak at around 216 nm and a positive peak at around 228 nm. Although the negative peak at around 216 nm has been used to assign the I-β conformation, a positive peak at around 228 nm is unknown. The reduced mean residue ellipticity observed for water-insoluble copolypeptides of Pro with Nva (No.8) has a $[\theta']$ of −6700 at 204 nm in HFIP. Poly(Pro, Leu) (No.9) was insoluble in water and HFIP, but soluble in dichloroethane and benzene; it has a Moffit parameter (b_0) of −170 in dichloroethane, which corresponds to a helix content of 27%.[14] The conformations of water-insoluble polymers No.8 and No.9 were very similar to that of the water-soluble polypeptides.

Structure and Solubility. We found that random copolypeptides comprising of L-proline and an L-amino acid with a small side chain, such as glycine or L-alanine, were soluble in water and occur in a collagen-like conformation. The solubility of copolypeptides in water increases by decreasing any hydrophobic in-

teractions between the side chains. However, a weak hydrophobic interaction between the side chains is not sufficient of the high solubility of polypeptides, since poly(Gly) is not soluble in water. We previously suggested that polypeptides that do not take an ordered conformation, such as an α-helix of a β-sheet, would be soluble in water. For disrupting the ordered conformation, proline is an ideal residue, due to its lack of an amide proton and the rigid nature of the backbone. In fact, copolypeptides of Pro with Gly, Ala or Abu were soluble in water. These non-ionic, but still water-soluble copolypeptides, are not only interesting from a structural view point, but will also find various clinical applications.

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References

1) A part of this work was presented at "the 60th National Meeting of Chemical Society of Japan, " Hiroshima, Oct. 1990, Abstr., 3C401.

2) C. H. Namford, A. Elliott, and W. E. Hanby, "Synthetic Polypeptides; The Synthesis of Polypeptides, " Academic Press, New York (1956), p. 28.

3) D. M. Seagel and W. Traub, *J. Mol. Biol.*, **47**, 87 (1969).

4) A. Yonath and W. Traub, *J. Mol. Biol.*, **43**, 461 (1969).

5) Y. Iizuka, T. Endo, and M. Oya, *Bull. Chem. Soc. Jpn.*, **47**, 1336 (1991).

6) J. Kurtz, A. Berger, and E. Katchalski, *Nature*, **178**, 1066 (1956); J. Kurtz, A. Berger, and E. Katchlski,"Recent Advances in Gelatin and Glue Research, " Pergamon Press, London (1958), p. 131.

7) J. Kurtz, G. Fasman, A. Berger, and E. Katchalski, *J. Am. Chem. Soc.*, **80**, 393 (1958).

8) A. Berger, J. Kurtz, and E. Katchalski, *J. Am. Chem. Soc.*, **76**, 5552 (1954).

9) M. Oya and R. Katakai,"Shin Jikken Kagaku Koza No. 19-1; Polypeptides, " ed by Nippon Kagaku Kai, Maruzen, Tokyo (1978), p. 244.

10) F. A. Bovey and F. P. Hood, *Biopolymers*, **5**, 325 (1967).

11) S. R. Snell and G. D. Fasman, *Biopolymers*, **11**, 1723 (1972).

12) G. Holzwarth, W. R. Gratzer, and P. Doty, *J. Am. Chem. Soc.*, **84**, 3194 (1962).

13) G. Holzwarth and P. Doty, *J. Am. Chem. Soc.*, **87**, 218 (1965).

14) G. T. Yang and P. Doty, *J. Am. Chem. Soc.*, **79**, 761 (1957).