Synthesis and the Solid-State Conformations of Cross-Linked Resin-Bound Oligo(leucine)s.^{1,2} Internal Aggregations through the Intermolecular Hydrogen Bonding of the Oligopeptides on the Polymer Networks

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ABSTRACT: Cross-linked and soluble polystyrene resin-bound oligo(leucine)s were prepared by the step-by-step coupling of Boc-Leu₃-OH to H-Phe-resins using dicyclohexylcarbodiimide and 1-hydroxy-1*H*-benzotriazole. The solid state conformational analysis of H{(Leu₃)_n}Phe-resins ($n = 1—5$, cross-linked resin; $n = 1—3$, soluble resin) by infrared spectroscopy indicated that the peptide chains had various conformations depending on the chain length: $n = 1$, essentially an unordered conformation; $n = 2—4$, mainly the β -structure; and $n = 5$, predominantly the α -helix. The formation of the β -structure indicates that resin-bound peptide chains can easily interact with each other through hydrogen bonds even in polymer networks. The correlation of the conformation in the swollen state with that in the solid state was examined using various solvents. Effects of shear stress on conformational transformations of H{(Leu₃)_n}Phe-resins ($n = 1—3$) was also examined.

KEY WORDS Conformation of Oligo(leucine)s / Cross-Linked Polystyrene Support / Fragment Condensation / Internal Aggregation / Shear Stress / Solid State / Soluble Polystyrene Support /

Conformations of monodispersed oligopeptides in solution³ and in the solid state⁴ have been extensively studied. A macromolecular C-terminal protecting group, poly(oxyethylene), was introduced into the peptide chemistry by Mutter *et al.*⁵ This group makes it possible to carry out conformational studies by commonly used methods such as circular dichroism (CD), nuclear magnetic resonance, and infrared (IR) spectroscopies, since the difficult solubility problems related to the numerous peptide sequences in aqueous and organic solvents can be solved by use of the poly(oxyethylene)-bound peptides. On the other hand, no conformational analysis of Merrifield resin-bound peptides has as yet been attempted, despite the fact that a study of the relationship between reactivities and conformations of resin-bound peptides in solid-phase peptide synthesis would provide significant results. Conformational studies of polystyrene

resin-bound oligopeptides in the solid state are also useful for investigating the relationship between conformations of resin-bound oligopeptides and physical properties, such as micro-phase-separation, of peptide-grafting polystyrenes. Solvation and swelling properties of peptide-containing cross-linked polystyrenes in solid-phase peptide synthesis were recently studied by Sarin *et al.*,⁶ who found that the space availability for peptide chain growth within swollen resin beads is not a limiting factor in peptide synthesis. This may be the case when higher internal aggregates are not formed between peptide chains bound to cross-linked polystyrene beads. In fact, it was recently reported that internal aggregation, due to the hydrophobicity of side-chain protecting groups, of peptide chains bound to cross-linked polyamide supports gives rise to additional cross-linking and reduces the accessibility of reactive groups within

the resin matrix to reagents and solvents.⁷

In this paper, we report the synthesis and conformational analysis of cross-linked and soluble resin-bound oligo(leucine)s in the solid state since the conformation of Boc-Leu_n-OCH₃ (*n*=2–7) in the solid and solution states have been well characterized by IR and CD measurements.^{3d–3f} We also present sound evidence that intraresin site separation of peptide chains does not occur in the solid state due to aggregation through intermolecular hydrogen bonding.

EXPERIMENTAL

General

The amino acid composition of acid hydrolysate was determined using a Shimadzu HPLC LC-3A all amino acid analysis system. IR spectra were measured on KBr disks using a JASCO IR spectrophotometer, model DS-701G. The KBr disks of peptide resins for Figures 1–3 were prepared by pressing a mixture of the peptide resin (10 mg) crushed loosely and the KBr (500 mg) pulverized, separately, using an agate-made mortar. The KBr

disks of peptide resins for Figures 4 and 5 were prepared as follows: the sample described above represents a weak-shear stressed sample; the weak-shear stressed sample was further pulverized for 10 min to give a medium-shear stressed sample; the medium-shear stressed sample was pulverized for an additional 10 min to give a sufficiently large shear stress.

Materials

Copoly(styrene–1% divinylbenzene) beads of 200–400 mesh, Bio-Beads S-X1, were purchased from Bio-Rad Laboratories. The H-Phe-resins were prepared according to the method described previously.^{8,9} Boc-Leu₃-OH was prepared by hydrolysis of Boc-Leu₃-OBzl using the method described before.¹⁰ The product was recrystallized from a mixture of ethanol and water, mp 155–158°C (lit.^{3a} 146–147°C). Found: C, 59.82; H, 9.48; N, 9.02%. Calcd for C₂₃H₄₃O₆N₃ · 0.5 H₂O: C, 59.20; H, 9.51; N, 9.00%.

Cross-linked Resin-Bound Oligoleucines

Five cycles of solid-phase peptide synthesis (the

Table I. Solid-phase peptide synthesis cycle for the addition of one oligopeptide^{a,b}

Function	Solvent and/or reagent (amount)	Time	Number of applications
		min	
1 Washing	CH ₂ Cl ₂	5	3
2 Peptide ^c	Boc-Leu ₃ -OH (3.0 eq) and HOBT ^d (3.3 eq)	30	1
3 Coupling ^c	DCC ^d (3.0 eq) in CH ₂ Cl ₂ (10 ml)	5 h ^c or 20 h ^c	1
4 Washing	CH ₂ Cl ₂	5	3
5 Washing ^c	C ₂ H ₅ OH and CH ₂ Cl ₂	5	3
6 Termination	Pyridine–acetic anhydride (1 : 1, v/v) (60 ml)	120	1
7 Washing ^c	CH ₂ Cl ₂ , C ₂ H ₅ OH, and CH ₂ Cl ₂	5	3
8 Deprotection	TFA ^d –CH ₂ Cl ₂ (2 : 3, v/v) (60 ml)	120	1
9 Washing ^c	CH ₂ Cl ₂ , C ₂ H ₅ OH, and CH ₂ Cl ₂	5	3
10 Neutralization	TEA ^d (1.0 g) in CH ₂ Cl ₂	10	2
11 Washing	CH ₂ Cl ₂	5	4

^a The resin used was 4.0 g, containing 0.324 mmol of Phe group. Coupling and neutralization reactions were performed in 60 ml of CH₂Cl₂.

^b The amount of each solvent used for washing was 60 ml.

^c Functions 2 and 3 were repeated twice. The first and second couplings were carried out for 5 h and 20 h, respectively.

^d See text.

^e Washing was carried out using each solvent.

cycle is described in Table I) were manually performed at room temperature, starting with 4.0 g of the H-Phe-resin. Following function 11 in Table I, an aliquot of the peptide resins was removed for IR spectroscopy measurement and amino acid analysis. The IR spectral samples of the peptides were prepared after being dried under vacuum at 45°C.

Soluble Resin-Bound Oligo(leucine)s

Soluble $H(\text{Leu}_3)_n\text{Phe-resins}$ ($n=1-3$) were synthesized on an aminomethylated polystyrene resin using the cycle described before.¹⁰ Methylene chloride was employed as the solvent in place of dimethylformamide. In the coupling reaction of the third cycle, gelation took place. The IR spectral samples of the soluble $H(\text{Leu}_3)_n\text{Phe-resins}$ ($n=1-3$) were prepared by precipitation with methylene chloride and ethanol followed by filtration, washing with ethanol, and drying under vacuum at 45°C.

RESULTS AND DISCUSSION

Assembly of the Test Peptides

Cross-linked resin-bound test oligopeptides having the general formula $H(\text{Leu}_3)_n\text{Phe-resins}$ ($n=1-5$) were synthesized on an aminomethylated copoly(styrene-1% divinylbenzene) resin support using the cycle summarized in Table I. The aminomethylated copoly(styrene-1% divinylbenzene) resin support⁸ has commonly been used for solid-phase peptide synthesis and contains no cluster of aminomethyl groups, as may be expected from the reaction mechanism. Synthesis of soluble $H(\text{Leu}_3)_n\text{Phe-resins}$ ($n=1-3$) was started with a soluble aminomethylated polystyrene resin.¹¹ The first residue, Phe, of the peptide chain was attached to the resin support through the oxymethyl-phenylacetamidomethyl group,⁹ and was used as an internal marker. The Phe content was $81 \mu\text{mol g}^{-1}$ of resin (*ca.* 1 mol% per styrene unit) for cross-linked resins and $104 \mu\text{mol g}^{-1}$ of resin for soluble resins according to amino acid analysis of the acid hydrolysates of the resins. The repeating Leu_3 unit was assembled by the step-by-step coupling of Boc-Leu₃-OH using dicyclohexylcarbodiimide (DCC) activation in the presence of 1-hydroxy-1*H*-benzotriazole (HOBt).¹² The amino acid protecting group was liberated from the Boc-peptide resin by

Table II. Amino acid analyses of $H(\text{Leu}_3)_n\text{Phe-resins}^a$

Sample	<i>n</i>	Amino acid analysis		Ratios of Leu/Phe	Recovery
		$\mu\text{mol g}^{-1}$ resin			of Phe
		Leu	Phe		%
Cross-linked resin	3	659	74	8.9	100
	5	899	67	13.4	96
Soluble resin	1	231	75	3.1	86
	2	475	81	5.9	92
	3	595	69	8.6	67
Cross-linked resin'	1	361	122	3.0	100
	2	604	101	6.0	87
	3	750	87	8.6	78
	5	1234	94	13.2	89

^a Hydrolyzed in 12 *M* HCl-propionic acid (1:2, v/v), 120°C, 66 h, in evacuated sealed tubes.

treatment with trifluoroacetic acid (TFA)/CH₂Cl₂ followed by triethylamine (TEA). Amino acid ratios were determined by hydrolysis and amino acid analysis of the peptide resins. The results are presented in Table II. The ratios of the $H(\text{Leu}_3)_n\text{Phe-resin}$ are very close to the calculated values, except that of the $H(\text{Leu}_3)_5\text{Phe-resin}$, which was 89% of the calculated value. This indicates that the average coupling yield of $H(\text{Leu}_3)_n\text{Phe-resins}$ ($n=3$ and 4) with Boc-Leu₃-OH was 83%. The results of the assembly of cross-linked resin-bound oligopeptides, which started with the H-Phe-resin having a Phe content of $127 \mu\text{mol g}^{-1}$ of resin, are also shown in Table II. In synthesizing the $H(\text{Leu}_3)_n\text{Phe-resins}$ ($n=1-3$), the termination reaction (Function 6 in Table I) was omitted and the double coupling reaction was carried out. For synthesis of the $H(\text{Leu}_3)_n\text{Phe-resins}$ ($n=4$ and 5), the coupling reaction was repeated three times, but this did not improve the Leu/Phe ratios.

Infrared Spectroscopic Conformational Analysis of Cross-Linked and Soluble Resin-Bound Oligo(leucine)s in the Solid State

Conformational analysis of cross-linked resin-bound oligopeptides, $H(\text{Leu}_3)_n\text{Phe-resins}$ ($n=1-5$), in the solid state was performed by IR spectroscopy. Figure 1 shows the important spectral regions for the conformational assignments (3600–3200

Figure 1. IR absorption spectra in the amide A region (a) and in the amide I and amide II regions (b) of the cross-linked $H(\text{Leu}_3)_n\text{Phe-resin}$ ($n=1-5$). Preparation of the IR spectral samples is described in the text. The absorption bands at 1603 cm^{-1} and 1585 cm^{-1} are due to the aromatic rings of the polystyrene supports.

cm^{-1} , amide A; $1700-1600\text{ cm}^{-1}$, amide I; $1600-1500\text{ cm}^{-1}$, amide II). The amide V region ($750-600\text{ cm}^{-1}$) was not considered since strong bands due to aromatic rings (polymer supports) are present in the same region. The $H\text{-Leu}_3\text{-Phe-resin}$ showed an essentially unordered conformation, characterized by a weak band at 3430 cm^{-1} and a strong band at 1665 cm^{-1} .^{3-5,13} The solid-state conformation of $H(\text{Leu}_3)_n\text{Phe-resins}$ ($n=2-4$) was predominantly the β -structure, judging from weak and strong bands at 3280 cm^{-1} , 1690 cm^{-1} , and $1632-1628\text{ cm}^{-1}$ accompanied by a weak or medium band at $1665-1655\text{ cm}^{-1}$, which was assigned to the unordered or α -helical conformation.^{3-5,13} When the chain was elongated from $n=4$ to $n=5$ of $H(\text{Leu}_3)_n\text{Phe-resin}$, the $\beta \rightarrow \alpha$ conformational transformation occurred. A similar observation was reported for oligopeptides free from macromolecular protecting groups.^{4,5} Thus, it may be said that the $H(\text{Leu}_3)_5\text{Phe-resin}$ formed predominantly the α -helical structure, on the bases of the appearance of weak and strong bands at 3340 cm^{-1} and 1655 cm^{-1} and the disappearance of the bands at 3280 cm^{-1} and 1630 cm^{-1} .^{3-5,13}

Figure 2 shows the amide I and amide II regions

($1700-1500\text{ cm}^{-1}$) of the IR spectra for the soluble $H(\text{Leu}_3)_n\text{Phe-resins}$ ($n=1-3$). It can be seen that the conformational behavior of the peptide chains bound to soluble resins is essentially the same as that of the cross-linked resin-bound oligopeptides when compared at similar peptide content. The same conformational behavior was observed for $H(\text{Leu}_3)_n\text{Phe-resins}$ ($n=1-3$ and 5 , cross-linked resin; $n=1-3$, soluble resin) in the swollen and solution states when methylene chloride, chloroform, tetrahydrofuran, and dioxane were used as the solvents.¹⁴ These findings suggest that the copoly(styrene-1% divinylbenzene) resin is not as rigid as expected¹⁵ and that the peptide chains of $H(\text{Leu}_3)_n\text{Phe-resins}$ ($n=2-4$) can easily interact intermolecularly with one another through hydrogen bonds even on the polymer networks. This interaction may give rise to additional cross-linking and influence the extent of swelling and reactivity of the terminal amino group. The low value of the amino acid ratio of the $H(\text{Leu}_3)_5\text{Phe-resin}$ (Table II) may be attributed to the limited accessibility of $\text{Boc-Leu}_3\text{-OH}$ to the coupling site.

In order to investigate whether the conformation in the swollen and solution states is maintained

Figure 2. IR absorption spectra in the amide I and amide II regions of the soluble $H(\text{Leu}_3)_n\text{Phe}$ -resins ($n=1-3$). Preparation of the IR spectral samples is described in the text. Assignment of the absorption bands at 1603 cm^{-1} and 1585 cm^{-1} , see Figure 1.

during the phase transformation to the solid state, further conformational analysis of the cross-linked $H(\text{Leu}_3)_n\text{Phe}$ -resins ($n=1$ and 2) and the soluble $H(\text{Leu}_3)_3\text{Phe}$ -resin was performed. Samples of $H\text{-Leu}_3\text{-Phe}$ -resins in the solid state were prepared by soaking them in carbon tetrachloride, benzene, or toluene overnight, filtering, washing with the respective solvent, and drying under vacuum at 45°C . $H(\text{Leu}_3)_n\text{Phe}$ -resins ($n=2$, cross-linked resin; $n=3$, soluble resin) were further swollen or dissolved in hexamethylphosphoramide. The swollen resins were shrunk with water, and the resin dissolved in hexamethylphosphoramide was precipitated with water. This was followed by filtering, washing in ethanol and drying the resins under vacuum at 45°C , and using them for IR measurement. The measurements were made on KBr disks and nujol mulls. The amide I region of the spectra obtained is shown in Figure 3. As in the swollen

state in carbon tetrachloride, benzene, or toluene,¹⁴ the conformation of the $H\text{-Leu}_3\text{-Phe}$ -resins in the solid state has β -like structure (Figure 3, curve 1), characterized by a sharp band at 3295 cm^{-1} and a strong band at 1640 cm^{-1} , accompanied by bands at 3440 cm^{-1} and 1670 cm^{-1} assignable to the unordered structure. However, the predominant conformation of the $H(\text{Leu}_3)_n\text{Phe}$ -resins ($n=2$, cross-linked resin; $n=3$, soluble resin) in the solid state is the β -structure (Figure 3, curves 2 and 3), in contrast to the fact that the $H(\text{Leu}_3)_n\text{Phe}$ -resins ($n=2$, cross-linked resin; $n=3$, soluble resin) in hexamethylphosphoramide are predominantly unordered and α -helical.¹⁴ The results obtained with the KBr disks and nujol mulls were essentially the same. These findings indicate that the cross-linked and soluble polystyrene resin-bound peptide chains aggregate with one another by internal hydrogen bonding in the solid state.

Figure 3. IR absorption spectra in the amide I region of the $H(\text{Leu}_3)_n\text{Phe-resins}$ ($n=1$ and 2 , cross-linked resin; $n=3$, soluble resin). Preparation of the IR spectral samples is described in the text. The $H\text{-Leu}_3\text{-Phe-resin}$ sample was prepared from carbon tetrachloride. For assignment of the absorption band at 1603 cm^{-1} , see Figure 1.

Since changes in the conformation of oligopeptides by application of shear stress were observed in the solid state,^{4c} the effects of shear stress on conformational changes of the $H(\text{Leu}_3)_n\text{Phe-resins}$ ($n=1$ and 2 , cross-linked resin; $n=3$, soluble resin) were investigated so as to clarify whether or not the conformations investigated here by KBr disks are the same as those before application of shear stress. When shear stress was applied to a sample of the $H(\text{Leu}_3)_3\text{Phe-resin}$, the intensity of absorption band at 1630 cm^{-1} decreased, while that at 1655 cm^{-1} increased (Figure 4). At the same time, the intensity of the absorption band at 3280 cm^{-1} decreased gradually with increasing intensity at $3400\text{--}3320\text{ cm}^{-1}$ (Figure 4). These results may be attributed to a partial $\beta \rightarrow \alpha$ and/or unordered conformational transformation by application of shear stress. The IR spectra in the amide A and amide I regions of the $H(\text{Leu}_3)_2\text{Phe-resin}$ following the application of shear stress are shown in Figure 5. The gradual increase in absorption at 3440 cm^{-1} and $1690\text{--}1660\text{ cm}^{-1}$ and the gradual decrease in absorption at 1630 cm^{-1} with increasing shear stress indicates a partial β -unordered confor-

Figure 4. IR absorption spectra of the soluble $H(\text{Leu}_3)_3\text{Phe-resin}$ in the amide A and amide I regions following application of shear stress: 1, weak-shear stressed sample; 2, medium-shear stressed sample; 3, sufficient-shear stressed sample.

Figure 5. IR absorption spectra of the cross-linked $H(\text{Leu}_3)_2\text{Phe}$ -resin in the amide A and amide I regions after application of shear stress: 1, weak-shear stressed sample; 2, medium-shear stressed sample; 3, sufficient-shear stressed sample.

mational transformation by shear stress.

The relationship between the conformation of cross-linked resin-bound oligopeptides and the reactivity of the terminal amino groups is now being investigated in relation to the solid-phase peptide synthesis by fragment condensation.

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