  Bull. Chem. Soc. Jpn., 62, 3577—3581 (1989)

Peptide Segment Separation by Tertiary Peptide Bonds.[1] Synthesis and Conformational Analysis of Cross-Linked Polystyrene Resin-Bound Human Proinsulin C-Peptide Fragments

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(Received June 5, 1989)

In order to clarify the role of tertiary peptide bonds in the conformational development of protected peptides in organic solvents, cross-linked polystyrene resin-bound human proinsulin C-peptide fragments were prepared by a fragment condensation method. For the purpose of clarifying the role of tertiary peptide bonds, it is indispensable to force coupling reactions to completion; the addition of HFIP in coupling reactions in CH_2Cl_2 was significantly effective for their completion when they did not proceed smoothly. The conformational behavior of resin-bound human proinsulin C-peptide fragments in CH_2Cl_2 was successively investigated using IR absorption spectroscopy. Two Pro residues forming tertiary peptide bonds played the role of stopping the development of helix and β-sheet structures in peptides, and the structure of N- and C-termini- and side-chain-protected human proinsulin C-peptide could be regarded as being assembled with peptide segment structures separated by the two Pro residues. When a peptide segment had a high helix-forming potential, a Pro residue in an N-terminal part of the peptide segment promoted its helix formation. β-Sheet structures formed by peptide segments in CH_2Cl_2 were easily disrupted by a strong electron-acceptor solvent of HFIP, suggesting that the β-sheet structure disruption of resin-bound peptides is important in achieving efficient coupling reactions.

In peptide and protein syntheses, sufficient solvation of a peptide chain is indispensable to achieve a coupling reaction quantitatively. As one means to accomplish sufficient solvation, we proposed "peptide segment separation by tertiary peptide bonds."[2] In fact, a tertiary peptide bond at a central position of peptide chains (Fig. 1) helps the sufficient solvation and remarkably improves peptide solubility. On the X–Pro and X–(Z)Y bonds in Fig. 1, X and Y are arbitrary amino acid residues and Z designates a suitable protecting group for the X–Y peptide bond,

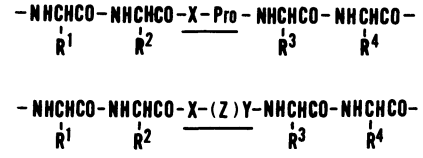

Fig. 1. Insertion of tertiary peptide bonds [X–Pro and X–(Z)Y] into central positions of peptide chains. An explanatory note for X, Y, and Z, see in the text.

which can be removed under certain conditions. Sufficient solvation and solubility improvements have been exactly attributed to the separation and disturbance of a β-sheet structure by rotation, due to a steric hindrance, of the tertiary peptide bond plane.

Here, it is significant to clarify the role of Pro and (Z)Y residues in the conformational development of protected peptides in organic solvents since the conformation of a peptide chain is closely related to sufficient solvation.[3–5] Although the prediction of conformations of protected peptides in organic solvents has been difficult so far, it is important in order to design synthetic routes for peptides and proteins without insolubility problems.[3] A Pro residue is well-recognized as being a strong helix and β-sheet breaker and plays the role in stopping the development of helix and β-sheet structures in native globular proteins.[6,7] Thus, tertiary peptide bonds formed by Pro and (Z)Y residues in protected peptides play the role of separating a peptide structure into various peptide segment structures. Namely, a peptide struc-

Boc-S(Bzl)LQ-OCH_2-Pam-resin 1

Boc-S(Bzl)LQPLALE(OBzl)GS(Bzl)LQ-OCH_2-Pam-resin 2

Boc-GGPGAGS(Bzl)LQPLALE(OBzl)GS(Bzl)LQ-OCH_2-Pam-resin 3

Boc-QVE(OBzl)LGGGPGAGS(Bzl)LQPLALE(OBzl)GS(Bzl)LQ-OCH_2-Pam-resin 4

Boc-QVGQVE(OBzl)LGGGPGAGS(Bzl)LQPLALE(OBzl)GS(Bzl)LQ-OCH_2-Pam-resin 5

Boc-E(OBzl)AE(OBzl)D(OBzl)LQVGQVE(OBzl)LGGGPGAGS(Bzl)LQPLALE(OBzl)GS(Bzl)LQ-OCH_2-Pam-resin 6

Fig. 2. Resin-bound human proinsulin C-peptide fragments **1**—**6** used in this study.

ture is regarded as being assembled with some of peptide segment structures separated by Pro and (Z)Y residues. We called the role "peptide segment separation by tertiary peptide bonds."[2]

In this study, we prepared resin-bound human proinsulin C-peptide fragments (Fig. 2) and examined their IR absorption spectroscopies in CH_2Cl_2 in order to demonstrate the role of Pro residues in the conformational development of protected peptides in CH_2Cl_2. In connection with efficient coupling reactions in solid-phase peptide synthesis by a fragment condensation procedure, we also investigated the disruption of the β-sheet structure of resin-bound human proinsulin C-peptide fragments in CH_2Cl_2 by the addition of a strong electron-acceptor solvent of HFIP.

Experimental

Materials. Copoly(styrene-1% divinylbenzene) beads of 200—400 mesh, Bio-Beads S-X1, were purchased from Bio-Rad Laboratories. The aminomethylation was carried out according to the method described previously.[8] Human proinsulin C-peptide fragments, Boc-Ser(Bzl)-Leu-Gln-Pro-Leu-Ala-Leu-Glu(OBzl)-Gly-OH, Boc-Gly-Gly-Pro-Gly-Ala-Gly-OH, Boc-Gln-Val-Glu(OBzl)-Leu-Gly-OH, Boc-Gln-Val-Gly-OH, and Boc-Glu(OBzl)-Ala-Glu(OBzl)-Asp(OBzl)-Leu-OH, were prepared before.[9] Boc-Ser(Bzl)-Leu-Gln-*p*-(oxymethyl)phenylacetic acid was obtained by hydrolysis of its trityl ester in a mixture of acetic acid, water, and methanol (5:1:4). The assembly of the peptide fragments was performed in CH_2Cl_2, substantially, according to the general procedures described previously.[10] The coupling reactions were carried out for 1 day using 3 equiv of carboxyl components, DCC, and HOBt, respectively. In the cycle for the peptide chain elongation, no termination reaction using pyridine and acetic anhydride was performed. Each coupling step was tested for completion by a ninhydrin test. When a test was positive after a double coupling reaction, a small amount of HFIP (2 vol% to CH_2Cl_2) was added, and the reaction was continued until the test became negative. Acid hydrolyses of the resin-bound peptides were performed with propionic acid/3 M (1 M=1 mol dm^{-3}) *p*-toluenesulfonic acid[11] (2/1, v/v) at 115 °C for 3 days. Amino acid analyses of the acid hydrolysates showed that the mole ratios of the component amino acid were close to the calculated values (Table 1).

IR Measurements. The IR absorption spectra of resin-bound peptides were recorded at room temperature with a JEOL Model JIR-100 FT-IR spectrometer. IR measurements in the swollen state were performed by holding the samples between potassium bromide windows after resin-bound peptides were swollen in CH_2Cl_2 or in CH_2Cl_2 containing HFIP at 0.1 M.

Results

Syntheses of Cross-Linked Polystyrene Resin-Bound Human Proinsulin C-Peptide. The copoly(styrene-1% divinylbenzene) resin support (Bio-Beads S-X1), used as a starting resin bead, has commonly been used for solid-phase peptide synthesis and the aminomethylated form is expected to have a uniform distribution of aminomethyl groups, based on the reaction mechanism of aminomethylation.[8] The aminomethyl content (76 μmol g^{-1} of resin) was selected in order to obtain rational information regarding the conformational development of peptide chains in the conventional solid-phase method.[12] The carboxyl component peptides were easily soluble in CH_2Cl_2 and were successively coupled with the aminomethylated polystyrene resin in CH_2Cl_2 using DCC and HOBt as coupling reagents.[13] In the preparation of resin-bound peptides **1—3**, coulping reactions proceeded smoothly without the addition of HFIP. On the other hand, in the preparation of peptides **4—6**, coupling reactions did not proceed smoothly, even at a double coupling reaction, and a ninhydrin test was positive at this stage. The addition of HFIP at this stage[14] was marvelously effective in forcing coupling reactions to completion and a ninhydrin test became negative while the reaction was continued for 24 h.

Conformations of Resin-Bound Human Proinsulin C-Peptide Fragments in CH_2Cl_2. The IR absorption spectra of resin-bound peptides **1—6** swollen in CH_2Cl_2 are presented in Fig. 3 in the most significant spectral regions for conformational assignments (3500—3100 cm^{-1}, amide A; 1800—1600 cm^{-1}, amide I). The results indicate the following: (1) resin-bound tripeptide **1** exhibits an unordered structure, characterized by appearance of medium and strong bands at 3425 and 1675 cm^{-1};[10,15] (2) resin-bound dodeca- and octadecapeptides **2** and **3** seem to form a helical structure, assigned by the appearance of medium and strong bands around 3310 and 1660 cm^{-1}, respectively,[10,15] accompanied by a small amount of a β-sheet structure, asigned by the appearance of a weak shoulder band at 1628 cm^{-1};[10,15] and (3) resin-bound tricosa-, hexacosa-, and hentriacontapeptides **4**, **5**, and

Table 1. The Results of Amino Acid Analysis of Resin-Bound Peptides **1—6**

Resin-bound peptide	Found (Calcd)							
	Asp	Ser	Glu	Pro	Gly	Ala	Val	Leu
1	—	1.00 (1)	1.00 (1)	—	—	—	—	1.00 (1)
2	—	1.95 (2)	2.85 (3)	0.94 (1)	1.13 (1)	1.13 (1)	—	3.89 (4)
3	—	1.81 (2)	2.89 (3)	2.19 (2)	4.92 (5)	2.19 (2)	—	3.84 (4)
4	—	1.99 (2)	4.89 (5)	2.07 (2)	6.11 (6)	2.14 (2)	0.92 (1)	4.88 (5)
5	—	2.18 (2)	6.44 (6)	2.21 (2)	6.86 (7)	1.89 (2)	1.82 (2)	4.60 (5)
6	1.06 (1)	1.88 (2)	7.86 (8)	2.15 (2)	7.17 (7)	3.24 (3)	1.78 (2)	5.96 (6)

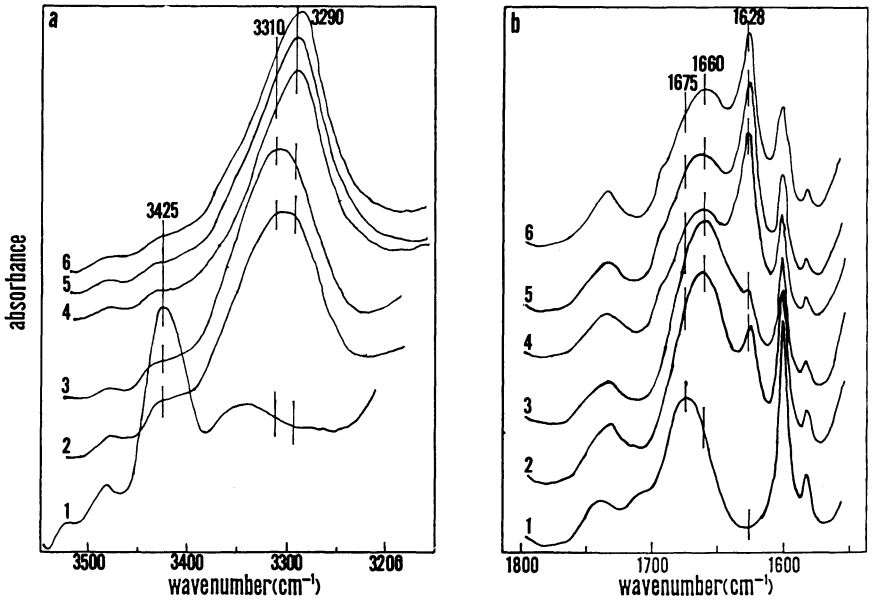

Fig. 3. IR absorption spectra (a) in the amide A and (b) in the amide I regions of resin-bound peptides **1**—**6** swollen in CH_2Cl_2. The absorption band at 1603 cm^{-1} is due to aromatic rings of the polystyrene support.

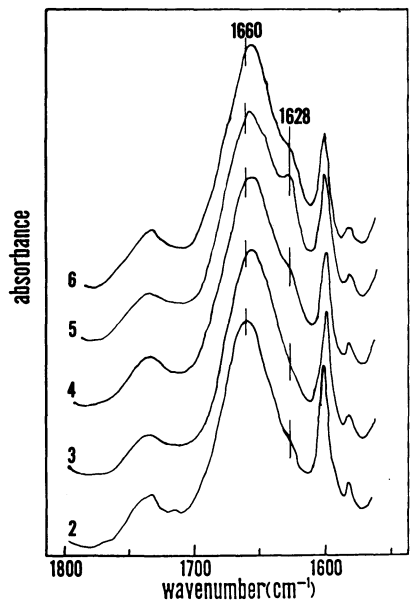

Fig. 4. IR absorption spectra in the amide I region of resin-bound peptides **2**—**6** swollen in CH_2Cl_2 containing HFIP at 0.1 M.

6 appear to have both helical and β-sheet structures, characterized by appearance of bands at 3310, 3290, 1660, and 1628 cm^{-1}, respectively.

In order to elucidate the role of HFIP in efficient coupling reactions in the solid-phase synthesis of human proinsulin C-peptide, conformational analysis of resin-bound peptides **2**—**6** in CH_2Cl_2 containing HFIP at 0.1 M was also performed using IR absorption spectroscopies (Fig. 4). The results showed that the β-sheet structure of resin-bound peptides **2**—**6** was sufficiently disrupted by a strong electron-acceptor solvent of HFIP, even at a low concentration of HFIP.

Discussion

The synthetic results of cross-linked polystyrene resin-bound human proinsulin C-peptide fragments clearly indicated that the addition of HFIP effectively forced coupling reactions in CH_2Cl_2 to completion due to the strong β-sheet structure-disrupting potential of HFIP.[16] Although solid-phase peptide synthesis using a fragment condensation procedure has been expected to be valuable for the synthesis of large peptides and proteins,[17] heterogeneous reaction conditions cause the formation of deletion and truncated sequences, and a drastic decrease in the coupling yields has been a serious obstacle.[18] In previous papers[19] we pointed out that the onset of a β-sheet aggregation by hydrogen bonding among pendant peptide chains brought about an additional cross-linking of the polymer network and that the coupling efficiencies of carboxyl component peptides with pendant peptides were remarkably decreased due to a restriction of their permeability into pendant peptides. The synthetic result, conformational analysis, and β-sheet-structure-disrupted behavior of resin-bound human proinsulin C-peptide fragments clearly indicate that the disruption of their β-sheet structures by the addition of HFIP is effective for the completion of coupling reactions in solid-phase peptide synthesis by a fragment condensation procedure and overcomes the serious obstacle of the drastic decrease in coupling yields.

Conformational analysis of resin-bound peptide **1**—**6** in CH_2Cl_2 also indicates that the concept of "the

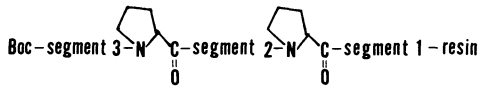

Fig. 5. The separation of human proinsulin C-peptide into three peptide segments by two Pro residues.

peptide segment separation by tertiary peptide bonds" is useful for estimating the conformational development of protected peptides in organic solvents, which is important for designing synthetic routes for peptides and proteins.[3] For example, the conformational behavior of peptides **4—6** suggests that the conformations of peptides **4—6** can be regarded as being assembled with three peptide segments separated by the two Pro residues (Fig. 5). With respect to conformations of peptides **4—6**, most parts of segment 1 and a part of segment 2, namely, Ser(Bzl)-Leu-Gln, are estimated to form a relatively stable helix, and N-terminal segments 3 of peptides **4—6** appear to form a relatively stable β-sheet structure although the conformation of segment 2 is not clear. These estimations were actually confirmed by conformational analyses of peptides **2** and **3**. The conformational behavior of resin-bound peptide **2** resembled that of Boc-Ser(Bzl)-Leu-Gln-Pro-Leu-Ala-Leu-Glu(OBzl)-Gly-OPac, which has a helical structure in CH_2Cl_2.[20] In the conformational development of peptide **2**, the Pro residue of resin-bound peptide **2** effectively suppresses the β-sheet formation of the octapeptide segment, Leu-Ala-Leu-Glu(OBzl)-Gly-Ser(Bzl)-Leu-Gln, due to the restriction of the backbone dihedral angles ϕ and ψ of a Pro residue,[4,5,21] and promotes its helix formation due to both the restriction of the backbone dihedral angles ϕ and ψ of a Pro residue[4,5] and the high helix-forming potential of peptide **2**.[20,22] For a prediction of the conformations of protected peptides in organic solvents, we have tentatively used the average helix, β-sheet, and coil conformation values, $<P_\alpha>$, $<P_\beta>$, and $<P_c>$,[3,20,23] which are determined by their amino acid compositions,[6] and demonstrated their validity. Practically, the $<P_\alpha>$ and $<P_\beta>$ values of segment 1 of peptides **2** are 1.06 and 0.92, respectively,[22] indicating the relatively high helix-forming potential of segment 1. The IR absorption spectrum of peptide **3** also shows little contribution of a β-sheet structure, indicating that the Pro residue of Boc-Gly-Gly-Pro-Gly-Ala-Gly- in resin-bound peptide **3** influentially distrurbs the onset of a β-sheet structure by the rotation of the tertiary peptide bond plane (Gly-Pro). A chain elongation of peptide **3** to peptide **4** appears to induce the onset of a β-sheet structure at the N-terminal segment 3 of peptide **4** and a further chain elongation of peptide **4** to peptide **5** seems to develop the β-sheet structure at N-terminal segment 3.[24] On the other hand, the β-sheet structure content of peptide **6** is lower than that of peptide **5**, probably due to the fact that peptide **6** is close to the critical chain length for a stable helix formation in CH_2Cl_2.

Due to the function that a Pro residue stops the development of helix and β-sheet structures in a peptide, conformational development at N-terminal segments 3 of peptides **4—6** in CH_2Cl_2 containing HFIP appears to occur regardless of segments 1 and 2. In the mixture, HFIP probably promotes a helical folding and/or a sufficient solvation of each peptide segment, depending on its conformational preference. The conformational development of protected peptides in organic solvents is strongly dependent on the nature of the organic solvents, especially their electron-donating and -accepting properties.[16] Thus, the disruption of the β-sheet structure of resin-bound peptides **2—6** by HFIP and by strong electron-donor solvents[22] suggests that the concept of "peptide segment separation by tertiary peptide bonds" is useful for estimating the conformational development of protected peptides in organic solvents having strong electron-donating and -accepting properties.

This work has been supported by a Grant-in-Aid from the Asahi Glass Foundation for Industrial Technology.

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1) The abbreviations for amino acids are those recommended by the IUPAC-IUB Commission on Biochemical Nomenclature, *J. Biol. Chem.*, **247**, 977 (1972). Amino acid symbols except for Gly denote the L-configuration. Additional abbreviations used are the following: HFIP, 1,1,1,3,3,3-hexafluoro-2-propanol; IR, infrared; Boc, *t*-butoxycarbonyl; Bzl, benzyl; Pam, phenylacetamidomethyl; DCC, dicyclohexylcarbodiimide; HOBt, 1-hydroxy-1*H*-benzotriazole.

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