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Design for the Peptide Analog of Calcium Binding Loops by Considering the Steric Restriction Effect of Incorporated Nonprotein Amino Acids

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A dodecapeptide was designed for a structural analog of calcium binding loops in EF-hand based on their averaged dihedral angles. The synthesized peptide contains three nonprotein amino acids, namely a D-alanine and two α-aminoisobutyric acids, which help this small segment to conserve the intrinsic conformation in calcium binding proteins by their steric restriction effect.

One of the essential factors for the next step of protein engineering is how to design amino acid sequence of de novo protein[1] which folds into tailor-made tertiary structure of its own accord. We expect that an utilization of nonprotein amino acids[2] enhances possibility for the construction of an artificial protein. Thus here, in order to demonstrate the steric restriction effect of nonprotein amino acids, the dodecapeptide containing three nonprotein amino acids was synthesized as a structural analog of calcium binding loop in EF-hand and the calcium binding activity of the peptide was investigated.

EF-hand[3] is the structural motif which is often found at the binding sites in various calcium binding proteins such as TnC, CaM, ICaBP, and Parv.[4] They are constructed with the common super secondary structure consisting of two α-helices and binding loop between helices. The sequences of the loops in various calcium binding proteins resemble each other and the tertiary structures of the several loops determined by X-ray studies also resemble.[5]

Several peptides corresponding to calcium binding loops and more extended sequences including the helical region have so far been synthesized.[6] The calcium binding activity of the synthetic peptide increased with the chain-length of the helical region. However, no remarkable calcium binding activity of the synthetic loops consisting of 12 amino acid residues was observed.[6] These results indicate that the chain-length of 12 amino acid residues is not enough for the segment to conserve intrinsic conformation.

In various calcium binding loops the amino acid residues at the positions 1, 3, 5, 6, 8, and 12 are conserved to a high degree; (i) Asp or Asn tends to be located at the positions 1, 3, and 5, (ii) Gly, Ile, and Glu tend to be located at the positions 6, 8, and 12, respectively. The α-carbonyl group of the amino acid residue at the positions 7 and the side chains of the amino acid residues at the positions 1, 3, 5, 9, and 12 coordinate to calcium cation octahedrally. However, the role of the amino acid residues at non-ligand positions which do

not coordinate to calcium cation directly, *i.e.*, the residues at the positions 2, 4, 6, 8, 10, and 11, have not been discussed much.

The averaged dihedral angles of the main chains of several calcium binding loops are listed in Table 1 with their standard deviation (SD).[7] The values of SD's at the non-ligand positions as well as at the ligand positions are small. In particular the values of SD's at the positions 6 and 8 are smaller than those at other non-ligand positions. This indicates that the tertiary structural homology of the residues at the positions 6 and 8 are high, which agrees with the sequential homology described above.

Table 1. Sequences and dihedral angles of several calcium binding loops

Position	Angle	Protein-loop										Average	SD
		3CPV-CD		3CPV-EF		3ICB-II		5TNC-III		3CLN-III			
1	φ	Asp	-70	Asp	-103	Asp	-67	Asp	-86	Asp	-76	-80	15
	ψ		77		55		88		80		62	72	14
	ω		-176		-174		179		-175		-178	-177	3
2	φ	Glu	-51	Ser	-59	Lys	-67	Lys	-64	Lys	-42	-57	10
	ψ		-66		-69		-61		-20		-33	-50	22
	ω		-169		178		179		-179		-179	-178	5
3	φ	Asp	-56	Asp	-47	Asn	-66	Asn	-106	Asp	-106	-76	28
	ψ		-6		-5		-52		3		30	-6	30
	ω		-170		-178		180		-179		-177	-177	4
4	φ	Lys	94	Gly	31	Gly	109	Ala	52	Gly	23	62	38
	ψ		34		79		23		48		59	49	22
	ω		-148		173		178		179		-179	-175	16
5	φ	Ser	-42	Asp	-114	Asp	-87	Asp	-104	Asn	-112	-92	30
	ψ		-6		-4		6		-1		-3	-2	5
	ω		-159		162		179		179		178	180	14
6	φ	Gly	77	Gly	86	Gly	79	Gly	94	Gly	99	87	9
	ψ		4		12		0		2		-12	1	9
	ω		-178		170		179		179		-177	179	5
7	φ	Phe	-162	Lys	-158	Glu	-130	Phe	-140	Tyr	-128	-144	16
	ψ		171		178		155		147		137	158	17
	ω		170		-170		178		173		176	177	8
8	φ	Ile	-97	Ile	-119	Val	-116	Ile	-101	Ile	-94	-105	11
	ψ		107		141		118		116		100	116	16
	ω		164		-175		176		-177		180	178	8
9	φ	Glu	-66	Gly	-131	Ser	-85	Asp	-94	Ser	-93	-94	24
	ψ		140		-117		175		174		175	-179	38
	ω		177		-154		-179		-177		-178	-174	12
10	φ	Glu	-39	Val	-46	Phe	-67	Ile	-61	Ala	-59	-54	12
	ψ		-96		-72		-34		-30		-41	-55	28
	ω		162		180		179		-179		178	176	8
11	φ	Asp	-22	Asp	-54	Glu	-64	Glu	-66	Ala	-58	-53	18
	ψ		-31		-76		-41		-47		-45	-48	17
	ω		-175		180		178		176		180	180	3
12	φ	Glu	-62	Glu	-81	Glu	-65	Glu	-61	Glu	-69	-68	8
	ψ		-66		-18		-38		-47		-51	-44	18
	ω		170		-177		179		179		179	178	5

The pair of the values of the averaged dihedral angles ϕ and ψ for the residue at position 8, $(\phi,\psi)^8$, is (-105,116). The pair belongs to β-sheet region in Ramanchandran map[8] of L-amino acid. Thus it is reasonable that Ile possessing high potential of β-sheet[9] is conserved at the position 8 in the sequential homology. The $(\phi,\psi)^6$ is (87,1), which is only allowed for Gly, not for L-amino acids, in Ramanchandran map.[8] Thus it is necessary that Gly is conserved at the position 6 in the sequential homology.

The $(\phi,\psi)^4$ is (62,49), which is classified to left-handed helix region.[8] Since D-amino acid is an preferable to L-amino acid for forming left-handed helix, we selected D-Ala for an appropriate residue at the position 4.[10] The $(\phi,\psi)^{10}$ and $(\phi,\psi)^{11}$ are (-54,-55) and (-53,-48), respectively. Both values are classified to α-helix region. We selected Aib for a suitable residue at positions 10 and 11, because Aib possesses high potential of α-helix owing to its steric restriction.[11]

According to above discussion, we designed the following sequence:

Asp-Lys-Asp-D-Ala-Asp-Gly-Tyr-Ile-Asp-Aib-Aib-Glu.[4]

The designed peptide was synthesized by the solid-phase method using an Applied Biosystems 430A peptide synthesizer. To avoid aspartimide formation, cyclohexyl ester was used as a protecting group for Asp.[12] The conditions of deprotection and purification were almost the same as those described previously.[13] The

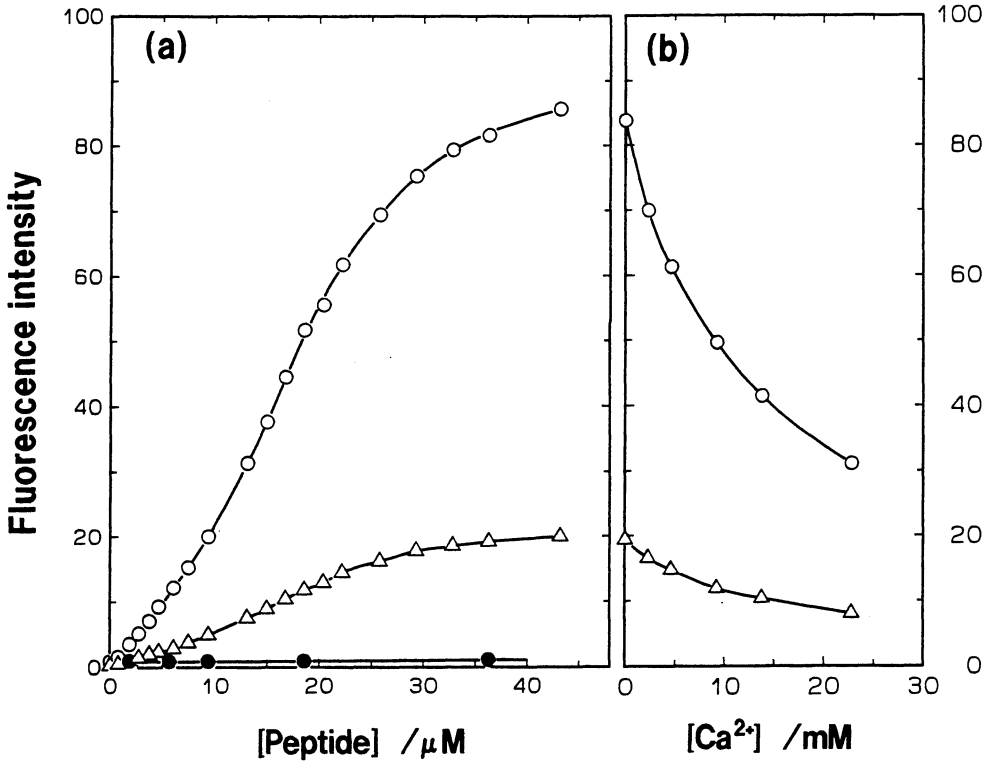

Fig. 1. (a): Terbium fluorescence at 544 nm (○) and 490 nm (△) as a function of the peptide concentration and fluorescence at 544 nm of the solution including the amino acid mixture corresponding to the peptide concentration (●). Terbium concentration was 21 μM in 5 mM HEPES (pH7.2). (b): Terbium removal by competitive binding of calcium.

purified peptide showed single peak in isocratic RP-HPLC and was identified by amino acid analysis after acid-hydrolysis.[14)]

Calcium-binding activity of the peptide was demonstrated by competitive binding between calcium and terbium ion. When terbium binds to the peptide, energy-transfer process occurs from excited Tyr residue to terbium ion,[15)] which results in characteristic luminescence at 490, 544, 585, and 621 nm. By monitoring the luminescence at 490 and 544 nm, calcium-binding constants were determined as 5.3×10^2 and 6.3×10^2 M^{-1} (1 M = 1 mol dm^{-3}), respectively (Fig. 1). Moreover the CD spectra of apo-peptide and metal-binding peptide were almost the same.

These results indicate that the conformation of the peptide resembles the intrinsic one in a calcium binding protein, in spite of its short chain-length, by the steric restriction effect of incorporated three non-protein amino acids. Thus, it is concluded that design based on dihedral angles and replacement with non-protein amino acids is effective to increase structural conservation of the isolated functional segment of a globular protein.

References

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2) Nonprotein amino acid, in this letter, is defined as the amino acid which does not have corresponding codon in biosynthesis.
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4) Abbreviations are as follows: TnC, troponin C; Parv, parvalbumin; ICaBP, vitamin D dependent intestinal calcium binding protein; CaM, calmodulin; Aib, α-amonoisobutyric acid.
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14) The result of amino acid analysis of the designed peptide is as follows: Asp 4.01(4), Glu 1.07(1), Gly 1.00(1), Ala 0.98(1), Aib 2.12(2), Ile 0.90(1), Try 0.91(1), and Lys 0.97(1).
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