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INFLUENCE OF AMINO ACID SEQUENCE ON THE FORMATION AND STABILITY OF CYCLOHEPTA-AMYLOSE (CYCLOMALTOHEPTAOSE) INCLUSION-COMPLEXES WITH PHENYLALANINE-CONTAINING DI-PEPTIDES

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ABSTRACT

The formation and the molecular dynamics of the inclusion complexes of cycloheptа-amylose (cyclomaltoheptaose) with L-lysyl-L-phenylalanine, L-phenylalanyl-L-leucine, and L-leucyl-L-phenylalanine in aqueous solution have been studied by ^{1}H- and ^{13}C-n.m.r. spectroscopy. The displacements in ^{1}H-chemical shift and the decrease in NT_1 η values for the guest carbons on addition of cycloheptа-amylose show that the inclusion complexes are formed by insertion of the guest's phenyl ring into the host's cavity, and the stability of the complexes depends on the types of amino acid residue adjoining the aromatic residue and on their sequence. Here, N is the number of protons directly bonded to a given carbon, T_1 is the ^{13}C spin–lattice relaxation-time, and η is the viscosity of the solution.

INTRODUCTION

The cycloamyloses (cyclodextrins) are cyclic oligosaccharides composed of at least six (1→4)-linked α-D-glucosyl residues. The cycloamylose has the shape of a hollow, truncated cone with primary and secondary hydroxyl-groups crowning the narrower and wider rims, respectively. The cycloamyloses can form inclusion complexes with a variety of guest molecules without any covalent bonds being formed, and, in some cases, they catalyse the reaction of included compounds[1–5]. Because of these properties, the cycloamyloses serve as models for studying topochemical aspects and catalytic reactions of enzymes. In particular, the cycloamyloses are models for such hydrolytic enzymes as esterase and protease in which the hydroxyl group of a catalytic serine residue attacks the acyl group of a bound substrate. It is well known that chymotrypsin, a typical serine protease, selectively includes the aromatic and bulky, non-polar side-chains of substrates into its nonpolar cavity.

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Therefore, it is relevant to investigate the molecular dynamics of inclusion complexes between cycloamyloses and aromatic amino acids and peptides as models for enzyme–substrate specific binding[6-9]. We now report on the formation and molecular dynamics of inclusion complexes of cyclohepta-amylose (cyclomaltoheptaose, β-cyclodextrin, β-CD) with the phenylalanine-containing dipeptides, L-lysyl-L-phenylalanine (Lys–Phe), L-phenylalanyl-L-leucine (Phe–Leu), and L-leucyl-L-phenylalanine (Leu–Phe).

EXPERIMENTAL

Materials. — β-CD and the dipeptides were purchased from Nakarai Chemicals, Ltd., Kyoto, and Sigma Chemical Co., Saint Louis, respectively; they were used without further purification. ^2H_2O, 40% NaO^2H solution in ^2H_2O, and 38% ^2HCl solution in ^2H_2O were purchased from Merck Sharp and Dohme, Canada, Ltd.

Methods. — ^1H- (100 MHz) and ^{13}C-n.m.r. (25 MHz) spectra were recorded with a JEOL JNM PS-100 spectrometer equipped with a PFT-100 pulse Fourier-transform system. Data were accumulated and processed in a JEOL JEC-100 computer. The ^{13}C spin–lattice relaxation-time (T_1) was measured by the inversion–recovery method using a 180°–t–90° pulse sequence, where t is the time interval between the 180° and 90° pulses. The estimated error in the ^{13}C-T_1 value was <15%. The ^1H- and ^{13}C-chemical shifts were referenced to external tetramethylsilane.

The macroscopic viscosity of the solution used in the ^{13}C-relaxation experiment was measured with a Cannon–Finske viscometer. U.v. spectra were recorded with a Beckman-25 spectrometer. The temperature was kept at 30 ± 2° for all experiments.

The pH values were read on a Toko Model TP-101 pH-meter with micro combination-electrode CE103, which enabled the measurement of pH values of solutions contained in an n.m.r. sample-tube.

RESULTS AND DISCUSSION

Association constant. — Association constants K_a for the complexation of β-CD wth dipeptides were determined from the changes in u.v. spectra of the dipeptides (concentration, 100μM) induced by the addition of 0→10mM β-CD[7], and by the displacements of ^{13}C-chemical shift for the dipeptides (concentration, 0.2M) on addition of 0→0.3M β-CD[8,9]. The K_a values in alkaline solutions could not be determined accurately on the basis of the u.v. spectra, because of an overlapping, large peak for alkoxide anion. The K_a values in acidic solutions also could not be determined by ^{13}C-n.m.r. spectroscopy, because of the poor solubility of β-CD. The K_a values determined for the β-CD–dipeptide systems by assuming a 1:1 complex, and by using a Hildebrand–Benesi relation[7-10], were ~180–300M^{-1} in acidic medium (p^2H 1.5) and ~50M^{-1} in alkaline medium (p^2H 13.0), which are compara-

ble with those for β-CD complexes with L-phenylalanine[8] and L-tyrosine[9]. The K_a values indicate that the equilibrium of the complexation reaction, β-CD + dipeptide ⇄ complex, lies predominantly on the complex side. The formation of inclusion complexes of the 1:1 type between β-CD and dipeptides was also confirmed by ^{1}H-n.m.r. measurements.

Changes in ^{1}H-chemical shift for β-CD and dipeptides induced by complex formation. — ^{1}H-N.m.r. spectroscopy can provide evidence for the inclusion of aromatic molecules into the cycloamylose cavity[11,12]. If inclusion occurs, H-3 and H-5 of the β-CD glucosyl residues, which are located in the cavity and directed toward its interior, should be shielded, because of the ring-current effect of the phenyl group. By contrast, if association takes place at the exterior of the torus, H-1, H-2, and H-4 should be more affected. As anticipated, upfield shifts of ~0.03 p.p.m. were observed for the H-3 and H-5 signals by the addition of a 1.3 molar excess of dipeptide, whereas the chemical shifts of the remaining proton resonances of β-CD were practically constant. These results are consistent with the inclusion of the phenyl ring of the L-Phe residue of the dipeptides. When the phenyl ring of the L-Phe residue is preferentially included into the cavity of β-CD, inclusion of the aliphatic side-chain of the other residue (L-Lys or L-Leu) of the dipeptide may be sterically prevented. Thus, complex formation involving inclusion of an aromatic side-chain into the cavity of β-CD was further confirmed by the observation of ^{1}H-chemical shifts of the aliphatic side-chain of the dipeptides. Significant shielding of the aliphatic protons of the side chain has been reported for linear dipeptides having one aromatic and one aliphatic amino acid, but was not found in dipeptides lacking an aromatic side-chain[13,14]. The side-chain protons of the L-Leu residue of the dipeptides Leu–Phe and Phe–Leu in the free state might be shielded to some extent by the neighbouring Phe aromatic residue; consequently, the L-Leu side-chain protons would exhibit lowfield shifts when that shielding effect is removed. Figs. 1 and 2 show plots of the side-chain ^{1}H-chemical shift of the L-Leu residue of Phe–Leu and Leu–Phe, in the absence and the presence of β-CD, as a function of p^{2}H. As can be seen, the ^{1}H-chemical shifts of the L-Leu side-chain of both dipeptides in the presence of β-CD are always greater (by ~0.5 p.p.m.) than those in the absence of β-CD over the acidic p^{2}H region. For Phe–Leu, the same relation also holds in the basic region. These results show the removal of the shielding effect of the Phe residue in the presence of β-CD, indicating clearly the inclusion of a phenyl ring into the cavity of β-CD.

For the L-Leu side-chain of Leu–Phe, ^{1}H-chemical shifts in the absence and the presence of β-CD become almost the same in the higher p^{2}H region. This result implies that the inclusion complex between β-CD and Leu–Phe becomes unstable at higher p^{2}H. The destabilisation of the complex in basic solution may be rationalised by consideration of the ionisation of the carboxyl terminal (pK_a 2) of the dipeptide and that of a secondary hydroxyl-group (pK_a 12) of β-CD. For the Leu–Phe–β-CD system, there is more electrostatic repulsion between the terminal carboxylate anion of Leu–Phe and the hydroxyl anion located on the wider rim of

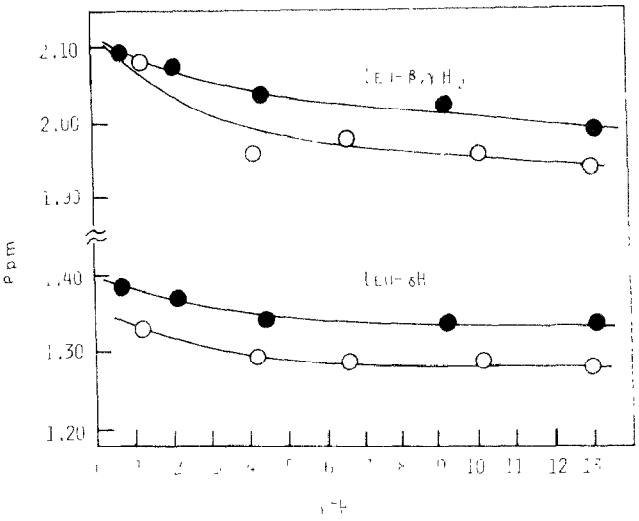


Fig. 1. ^{1}H-Chemical shifts of the L-Leu residue of Phe–Leu, in the absence (—○—) and the presence of equimolar β-CD (—●—), as a function of p^{2}H.

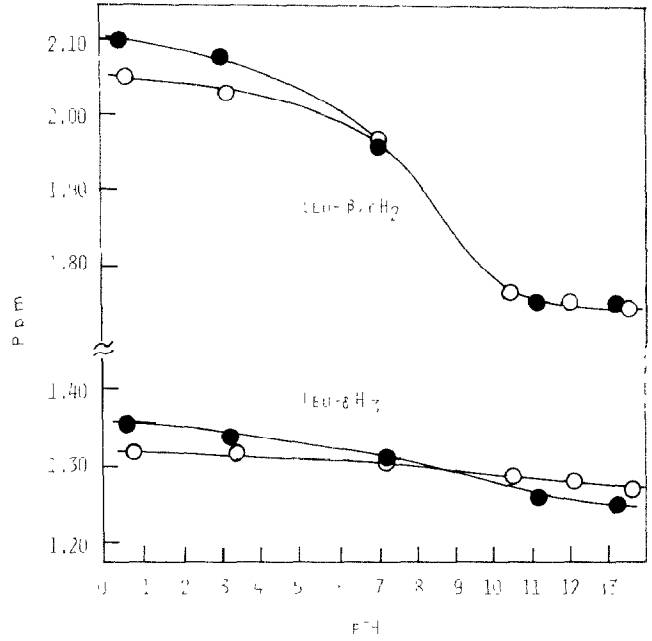


Fig. 2. ^{1}H-Chemical shifts of the L-Leu residue of Leu–Phe, in the absence (—○—) and the presence of equimolar β-CD (—●—), as a function of p^{2}H.

β-CD when the phenyl ring of dipeptide is included into the cavity of β-CD at high pH. This repulsive interaction prevents the formation of a stable inclusion-complex.

The existence of repulsive electrostatic interaction was indicated by observation of the conformational change of the Phe side-chain[15]. The ^{1}H resonances of

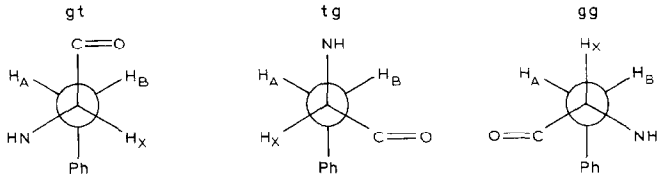
the α- and β-protons of L-Phe in Leu–Phe could be analysed as an ABX three-spin system. Table I contains the coupling constants and the rotamer populations estimated by use of the Feeney approximation[16]. In the free and the complexed states, the gt rotamer (aromatic ring trans to the carboxyl groups) is the most stable. On complex formation the gt-rotamer population increases in alkaline solution, whereas the change of rotamer population is insignificant in neutral solution. In alkaline solution, the repulsive electrostatic interactions between the ionised hydroxyl-groups and the carboxylate group of the guest keep the latter away from the former, *i.e.*, the gt rotamer becomes more stable. For the Phe–Leu–β-CD complex, in which the phenyl ring of the amino-terminal Phe residue is included, the carboxylate anion is situated too far away from the hydroxyl anion of the β-CD for severe interaction.

TABLE I

^{1}H-COUPLING CONSTANTS AND ROTAMER POPULATIONS FOR THE L-Phe RESIDUE OF Leu–Phe AND ITS MOLECULAR INCLUSION COMPLEXES WITH β-CD

Compounds	p^2H	*Coupling constants (Hz)*			*Rotamer populations*[a]		
		J_{AB}	J_{AX}	J_{BX}	*gt*	*tg*	*gg*
[Leu–Phe]	7.2	13.8	8.6	5.4	0.61	0.31	0.08
[Leu–Phe, β-CD]	7.2	13.9	8.7	5.6	0.61	0.33	0.06
[Leu–Phe]	12.6	13.4	8.4	5.3	0.58	0.29	0.13
[Leu–Phe, β-CD]	13.0	13.8	8.8	5.3	0.63	0.28	0.09

[a]Calculated by Feeney's approximation[16]. Rotamer notations are as follows.



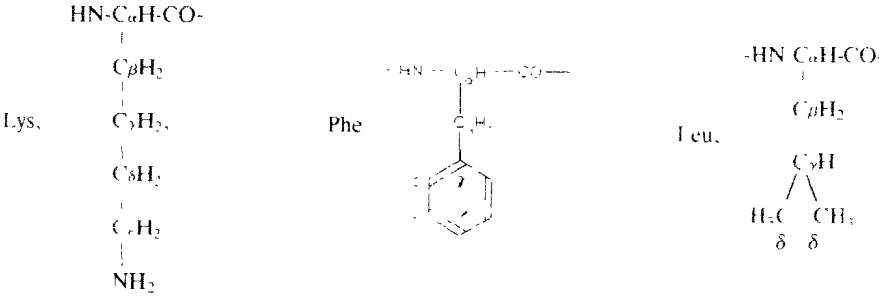
Dynamic aspects of β-CD inclusion-complexes with dipeptides. — Measurements of ^{13}C-T_1 values are particularly useful for investigation of the molecular dynamics of cycloamylose inclusion-complexes[6–9]. If inclusion complexes are formed, molecular motion must be restricted. In this paper, we have adopted the $NT_1\eta$ value as a measure of the effect of complex formation on molecular motion. Here, N is the number of protons directly bonded to a given carbon, and η is the viscosity of the solution. The use of the $NT_1\eta$ value has been estalished[8,9]. According to the theory of magnetic relaxation, assuming Brownian molecular-motion, a decrease in $NT_1\eta$ value corresponds to an increase in molecular volume. For cycloamylose inclusion-complexes, the greater the dynamic coupling between the guest and cycloamylose, the greater is the increase in the apparent molecular volume of each component and hence the greater is the decrease in the $NT_1\eta$ value. Thus, the

TABLE II

VALUES OF $NT_1\eta$[a] FOR GUEST DIPEPTIDES AND THEIR COMPLEXES WITH β-CD

Compounds[b]	*Viscosity (η) (cp)*	$NT_1\eta$ (s.cp)									
		Phe-α	*Phe-β*	*Phe-δ*	*Phe-ε*	*Phe-ζ*	*Lys-α*	*Lys-β*	*Lys-γ*	*Lys-δ*	*Lys-ε*
[Lys–Phe][c]	1.58	0.49	1.15	1.29	1.24	0.65	0.79	0.79	1.84	2.10	2.63
[Lys–Phe, β-CD][d]	3.54	0.39	0.96	0.82	0.78	0.41	0.52	0.72	0.76	0.74	0.94
		Phe-α	*Phe-β*	*Phe-δ*	*Phe-ε*	*Phe-ζ*	*Leu-α*	*Leu-β*	*Leu-γ*	*Leu-δ*	*Leu-δ*
[Phe–Leu][c]	1.60	0.78	1.40	1.47	1.45	1.13	0.90	0.92	1.40	5.70	5.67
[Phe–Leu, β-CD][d]	3.42	0.81	0.88	0.48	0.53	0.36	0.79	0.91	1.11	5.13	5.22
		Phe-α	*Phe-β*	*Phe-δ*	*Phe-ε*	*Phe-ζ*	*Leu-α*	*Leu-β*	*Leu-γ*	*Leu-δ*	*Leu-δ*
[Leu–Phe][c]	1.65	0.67	1.22	1.47	1.44	1.02	0.90	1.02	1.39	5.31	5.42
[Leu–Phe, β-CD][d]	3.50	0.70	0.91	1.11	1.13	0.77	0.65	0.82	1.07	5.12	5.34

[a] T_1 is the ^{13}C spin–lattice relaxation-time, N is the number of protons attached to the carbon, and η is the solution viscosity. [b] Assignments of carbon atoms for Lys, Phe, and Leu are as follows.



[c] p^2H 12.5, [Dipeptide] = 0.2M. [d] p^2H 13.0, [Dipeptide] = 0.2M, [β-CD] = 0.2M

extent of the decrease in $NT_1\eta$ value may be used as a measure of the strength of dynamic coupling of dipeptides with β-CD.

Table II contains $NT_1\eta$ values for dipeptides and the values of the solution viscosity measured in alkaline solutions. The $NT_1\eta$ values for aromatic carbons Cδ and Cε of L-Phe residues of dipeptides in the free and the complexed states are larger than those of Cζ. These results indicate the existence of rapid, internal rotation of the phenyl ring even in the complexed state, since an internal rotation that is faster than the overall molecular motion increases the NT_1 value[17].

Table III contains the ratios of the $NT_1\eta$ values for the complexed and free states of the dipeptides. The smaller value of this ratio corresponds to the restriction of molecular motion of the guest molecule by complexation with β-CD. All of the $NT_1\eta$ values for dipeptides greatly decrease on complex formation with β-CD, as expected; *i.e.*, the molecular motions of dipeptides were greatly restricted by complexation. The aromatic-ring carbons of the L-Phe residue in each β-CD–dipeptide system show a larger decrease in the $NT_1\eta$ values than those for carbons Cα and Cβ in the same dipeptides, indicating a larger slow-down of the internal rotation of the phenyl ring than for the other part, or an overall molecular reorientation. The results clearly show the formation of inclusion complexes of the guest dipeptides with the host β-CD by insertion of the phenyl ring into the cavity of β-CD.

For three dipeptides, the ratios $(NT_1\eta)_{complex}/(NT_1\eta)_{free}$ for the aromatic carbons are in the order Leu–Phe > Lys–Phe > Phe–Leu. This result implies that the amino-terminal L-Phe residue is more strongly fixed in the cavity of β-CD than the carboxylate-terminal residue. In other words, Phe–Leu forms a more stable inclusion-complex than Lys–Phe or Leu–Phe. This is consistent with the results obtained from measurements of ^{1}H-chemical shift. The two methyl carbons, Cδ, of the side chain of the L-Leu residue in Leu–Phe and Phe–Leu show high values of the $NT_1\eta$ ratios, indicating that these carbons retain high moility even after the complexation. Upon complex formation between Lys–Phe and β-CD, on the other hand, the $NT_1\eta$ ratios of side-chain carbons, Cδ and Cε, of the L-Lys residue in

TABLE III

VALUES OF $NT_1\eta^a$ RATIOS FOR THE COMPLEXED AND FREE STATES OF DIPEPTIDES

Compounds	$(NT_1\eta)_{complex}/(NT_1\eta)_{free}$									
	Phe-α	*Phe-β*	*Phe-δ*	*Phe-ε*	*Phe-ζ*	*Lys-α*	*Lys-β*	*Lys-γ*	*Lys-δ*	*Lys-ε*
[Lys–Phe]	0.80	0.83	0.64	0.63	0.63	0.66	0.91	0.41	0.35	0.36
	Phe-α	*Phe-β*	*Phe-δ*	*Phe-ε*	*Phe-ζ*	*Lys-α*	*Leu-β*	*Leu-γ*	*Leu-δ*	*Leu-ε*
[Phe–Leu]	1.04	0.63	0.33	0.37	0.32	0.88	0.99	0.79	0.90	0.92
[Leu–Phe]	1.04	0.75	0.76	0.78	0.75	0.72	0.80	0.77	0.96	0.99

[a] T_1 is the ^{13}C spin–lattice relaxation-time, N is the number of protons attached to the carbon, and η is the solution viscosity. The corresponding $NT_1\eta$ values are given in Table II. See also the footnotes of Table II.

Lys–Phe are extremely small. In the free state, the $NT_1\eta$ values show a higher mobility for these carbons as compared with other carbons in Lys–Phe. This result might suggest the existence of further interaction between the Lys side-chain end and β-CD in the complexed state. The most likely interaction is hydrogen bonding between the $N_\varepsilon H$ of the Lys residue and the hydroxyl group of β-CD. The decrease in the mobility of the Lys side-chain may be due to the anchoring effect of the hydrogen bond at the end of the Lys side-chain on the molecular motion[18]. In any case, such secondary interaction contributes to the stability of the Lys–Phe–β-CD complex, since the decrease of the $NT_1\eta$ ratios for the aromatic carbons in Lys–Phe are larger than those in Leu–Phe.

The formation of inclusion complexes between β-CD and the phenylalanine-containing dipeptides investigated here shows some characteristics of an enzyme–substrate complexation. The host β-CD has a hydrophobic cavity that is suitable for specific binding of the phenyl ring. The hydrophobic interaction seems to be the most probable driving-force for the formation of a stable complex, and additional interactions, occurring out of the cavity, make the complex stable or unstable depending on the nature of these interactions. The Leu–Phe–β-CD complex, which is stable in acidic solution, becomes unstable in alkaline solution because of an electrostatic, repulsive interaction between the dipeptide and β-CD. By the contribution of hydrogen bonding of the Lys side-chain, the Lys–Phe complex is more stable than the Leu–Phe complex in alkaline solution. Of the three dipeptides, Phe–Leu forms the dynamically most-stable complex with β-CD in alkaline solution because of the lack of repulsive interactions. Thus, the stability of cycloamylose inclusion-complexes with dipeptides varies with both the type of amino acid residue adjoining the aromatic residue and their sequence. It is concluded that cycloamylose can recognise the amino acid sequence as can an enzyme, although the recognition ability of the former is significantly weaker than the latter. It was confirmed that cycloamylose also has the ability to select the conformation of guest compounds at the time of formation of inclusion complexes. Consequently, it can be said that cycloamylose is a good, but not the best, mimic of enzymes.

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