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A ^{13}C-N.M.R. STUDY OF THE FORMATION AND MOLECULAR DYNAMICS OF CYCLOAMYLOSE INCLUSION-COMPLEXES WITH AROMATIC AMINO ACIDS

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ABSTRACT

The formation and the molecular dynamics of the inclusion complexes of host cyclohexa- and cycloheptа-amyloses with guest L-tyrosine in alkaline solution have been studied by ^{13}C-n.m.r. spectroscopy and the results compared with those for guest L-phenylalanine. The displacement of ^{13}C-chemical shift and the decrease in $NT_1\eta$ for the guest L-tyrosine carbons on addition of cycloheptа-amylose show that they form the stable inclusion-complex by insertion of the guest's aromatic ring into the host's cavity, and the strength of the dynamic coupling is the same as that between L-phenylalanine and cycloheptа-amylose. 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. In contrast to L-phenylalanine, L-tyrosine could not form an inclusion complex with cyclohexa-amylose.

INTRODUCTION

The cycloamyloses (cyclodextrins) are cyclic oligosaccharides that contain at least six (1→4)-linked α-D-glucose residues. Cycloamylose has the shape of a hollow, truncated cone with primary and secondary hydroxyl groups crowning opposite ends of the torus. The inside and outside of the cycloamylose cavity are relatively hydrophobic and hydrophilic, respectively. An important property of cycloamyloses is their ability to admit various guest molecules into the hydrophobic cavity, without any covalent bonds being formed[1–3]. Because of this property, the cycloamyloses serve as models for studying topochemical aspects and catalytic reactions of enzymes. Cycloamylose inclusion-complexes are also utilised in a large variety of fields[3].

It is relevant to investigate the molecular dynamics of inclusion complexes between cycloamyloses and aromatic amino acids as models for enzyme–substrate specific binding. It is well known that chymotrypsin, a typical serine protease, selectively cleaves peptide bonds on the carboxyl side of residues having aromatic side-

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chains and of bulky, hydrophobic residues. In these enzymic reactions, side chains of substrates are inserted into a non-polar pocket in the enzyme[4].

In preceding papers[5,6], the molecular dynamics of the inclusion complexes of cyclohexa-, cyclohepta-, and cycloocta-amyloses (α-, β-, and γ-cyclodextrins; or α-CD, β-CD, and γ-CD) with L-phenylalanine (L-Phe) in aqueous solution at different pH values have been studied by ^{13}C-n.m.r. spectroscopy. It was found that the strength of the dynamic coupling between cycloamylose and L-phenylalanine depends on the cavity size of the host and the pH of the medium. The strongest coupling was observed for the cyclohepta-amylose–L-phenylalanine system at p^{2}H 11.3, where the phenyl ring is deeply and tightly contained in the cavity.

We now report on the formation and molecular dynamics of inclusion complexes of α-CD and β-CD with L-tyrosine (L-Tyr) in alkaline solution. The influence of the hydroxyl group of L-tyrosine on the formation of the complex may be assessed by comparing the results with those obtained for cycloamylose–L-phenylalanine systems. Determination of the geometry of the cycloamylose–substrate complexes as well as the tightness of complex formation is essential for an understanding of the mechanism of enzymic catalysis of the cycloamyloses.

EXPERIMENTAL

Materials. — Cyclohexa- and cyclohepta-amylose and L-tyrosine were purchased from Nakarai Chemicals, Ltd., Kyoto. The $[\alpha]_D^{25}$ values of +150° and +162°, respectively, agreed with those previously reported[2]. ^2H_2O (isotopic purity, 99.7 atom%) and 40% NaO^{2}H solution in ^2H_2O were purchased from Merck Sharp and Dohme Canada Ltd.

Methods. — ^{13}C-N.m.r. spectra were recorded with a JEOL JNM PS-100 spectrometer (25 MHz) equipped with a PFT-100 pulse Fourier-transform system and proton-noise decoupler. Data were accumulated in a JEOL JEC-100 computer, using 4000-Hz spectral widths and 4096 data points (resolution 2.0 Hz). The 90° pulse, requiring 20 μs, was used to accumulate the interferograms with a repetition time at least five times longer than the longest ^{13}C spin–lattice relaxation-time (^{13}C-T_1) to be measured. The spin–lattice relaxation-times were 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 ^{13}C-T_1 was less than $\pm$10%. The concentrations of cycloamyloses were 0.24M, which were 1.2 times larger than those of L-tyrosine (0.20M) in the measurements of T_1 of L-tyrosine. ^{13}C-Chemical shifts were measured in p.p.m. downfield from external tetramethylsilane.

The macroscopic viscosity of the solution used in the ^{13}C-relaxation experiment was measured with a Cannon–Finske viscometer. The temperature was kept at 34 $\pm$1° for all experiments.

RESULTS AND DISCUSSION

Cycloamylose-induced, ^{13}C-chemical shift changes in L-*tyrosine*

All peaks in the ^{13}C spectra of both cycloamyloses and L-tyrosine have been assigned[5]. As found previously, the spectrum of each cycloamylose–L-tyrosine system consists of only one set of peaks corresponding to the components. The displacements of ^{13}C-chemical shifts for L-tyrosine on addition of β-CD in M NaO^2H are shown in Fig. 1. In this experiment, the concentration of L-tyrosine was held constant at 0.20M and the concentration of β-CD was varied between 0 and 0.40M. All carbons, except for C_ζ of L-tyrosine, show induced shifts with comparable magnitudes and the same signs as those of L-phenylalanine in M NaO^2H. The C_ζ of L-tyrosine, to which a hydroxyl group is linked, shows a smaller chemical-shift displacement (with opposite sign) than that of L-phenylalanine. It is not easy to interpret the cause of the magnitudes and directions of the induced shifts[7].

From plots of chemical-shift displacement *vs* molar ratio [β-CD]/[L-Tyr], it is possible to determine the value of the association constant K_a for a 1 : 1 complex by using modified Hildebrand–Benesi equations[6,8,9]. The K_a value for the β-CD–L-tyrosine system in M NaO^2H was estimated to be $\sim 10^2$–10^3 M^{-1}, which was compar-

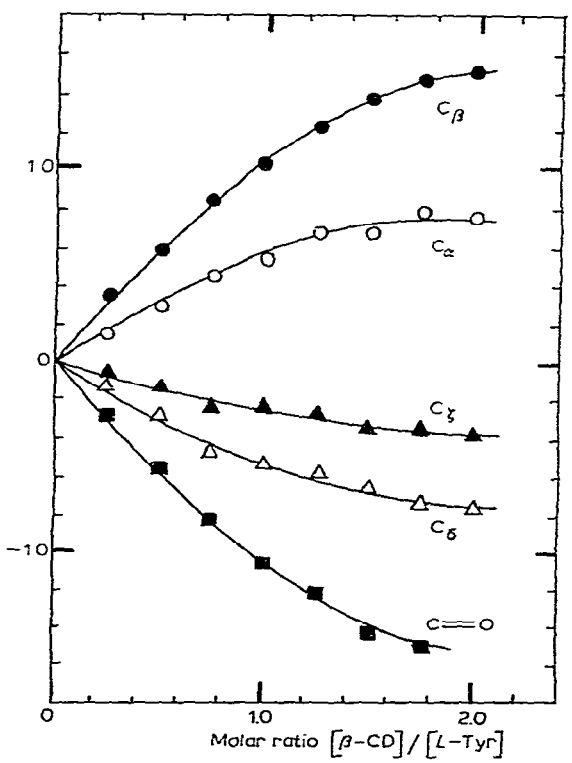


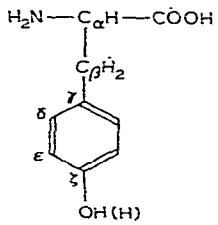
Fig. 1. ^{13}C Chemical-shift displacements ($\Delta\delta$) of L-tyrosine carbons on addition of β-CD in M NaO^2H, as a function of the molar ratio [β-CD]/[L-Tyr]. Negative signs indicate upfield dispiacements.

TABLE I

^{13}C SPIN–LATTICE RELAXATION-TIME (T_1; S) FOR L-TYROSINE, L-PHENYLALANINE, AND THEIR COMPLEXES WITH α-CD AND β-CD IN M NaO^2H[a]

Compound[b]	*Carbon*				
	α	β	δ	ε	ζ
[*L-Tyr*]	0.89	0.60	1.07	1.17	
[*L-Tyr*, α-CD]	0.42	0.20	0.50	0.52	
[*L-Tyr*, β-CD]	0.17	0.10	0.15	0.14	
[*L-Phe*]	1.41	0.74	1.91	1.87	1.38
[*L-Phe*, α-CD]	0.56	0.35	0.61	0.60	0.39
[*L-Phe*, β-CD]	0.20	0.21	0.29	0.26	0.17

[a]Concentration: L-Tyr and L-Phe, 0.20 mol/dm^3; α-CD and β-CD, 0.24 mol/dm^3. [b]Assignments of carbon atoms for L-*Tyr* (L-Phe) are as follows:



able with the K_a value of $\sim 10^3$ M^{-1} for the β-CD–L-phenylalanine complex in alkaline solution determined in a similar way[6]. The displacements in ^{13}C-chemical shifts induced in L-tyrosine spectra by addition of α-CD were less than 0.20 p.p.m. even at molar ratio [α-CD]/[L-Tyr] = 2, and the molar-ratio dependences of displacements showed some fluctuation over a digital resolution. Thus, we could not estimate the K_a value for the α-CD–L-tyrosine system. The small displacements in chemical shift suggest that the forces which induce the complexing of α-CD with L-tyrosine are weak or that there is no detectable interaction.

^{13}C *Spin–lattice relaxation-times for* L-*tyrosine*

Table I contains the values of ^{13}C spin–lattice relaxation-times for L-tyrosine in free and cycloamylose–L-tyrosine mixed systems in M NaO^2H. For comparison, the values for L-phenylalanine are also shown in Table I.

In the limit of the rapid exchange process of eq. *1*,

$$CD + S \rightleftarrows [CD,S] \qquad (1)$$

where CD, S, and [CD,S] are cycloamylose, substrate amino acid, and cycloamylose–substrate complex, respectively, an average relaxation-rate $1/T_1$, given by eq. *2*, is measured.

$$\frac{1}{T_1} = P_f \frac{1}{T_1(f)} + P_c \frac{1}{T_1(c)} \qquad (2)$$

Here, $T_1(f)$ and $T_1(c)$ are intramolecular, spin–lattice relaxation-times for a ^{13}C spin in the free and complexed states, and P_f and P_c ($= 1 - P_f$) are the probabilities that CD or S are found in the free and complexed states, respectively. The observation of only one set of ^{13}C resonances indicates that the complexing of cycloamylose with substrate is a rapid process of the ^{13}C-n.m.r. time-scale. For the process in eq. *1*, the values P_c and P_f can be estimated from the association constants[5]. In our experimental conditions for measurement of ^{13}C-T_1 (the K_a value is $>10^2$ M^{-1}, and the molar ratio [β-CD]/[S] is 1.2), the value P_c is estimated to be >0.95. Thus, we can regard the T_1 values of L-tyrosine and L-phenylalanine observed in β-CD–L-tyrosine and β-CD–L-phenylalanine mixtures as exclusively those of the complexed states.

Dynamic aspects of cycloamylose inclusion-complexes

For a rapidly rotating molecule of medium size, the ^{13}C relaxation mechanism of the protonated carbon nucleus seems to be governed predominantly by ^{13}C–^1H dipole–dipole interaction. In this case the NT_1 value, where N is the number of directly bonded protons, depends on the shape and size of molecule and the viscosity of solution. For example, the NT_1 value for a molecule undergoing isotropic reorientation is given[10,11] by eq. *3*.

$$1/NT_1 = \hbar^2\gamma_C^2\gamma_H^2 r_{CH}^{-6}\eta f_r V_m/kT, \qquad (3)$$

where k is Boltzmann's constant, T is the temperature in Kelvin, η is the viscosity of the solution, f_r is a microviscosity factor, V_m is the molecular volume of spherical top, r_{CH} is the carbon–hydrogen bond-length, and γ_H and γ_C are the gyromagnetic ratios of ^1H and ^{13}C nuclei. According to eq. *3*, the $NT_1\eta$ value for a given ^{13}C atom must be constant when all factors except the solution viscosity remain unchanged. The expected constancy has been observed for $NT_1\eta$ values of L-phenylalanine in alkaline

TABLE II

VALUES OF $NT_1\eta$[a] FOR L-TYROSINE, L-PHENYLALANINE, AND THEIR COMPLEXES WITH α-CD AND β-CD IN M NaO^2H

Compounds	*Viscosity (cp)* (η)	$NT_1\eta$				
		Carbon α	β	δ	ε	ζ
[*L-Tyr*]	1.33	1.18	1.60	1.42	1.56	
[*L-Tyr*, α-CD]	2.69	1.13	1.08	1.35	1.40	
[*L-Tyr*, β-CD]	3.27	0.55	0.62	0.50	0.47	
[*L-Phe*]	1.35	1.90	2.00	2.58	2.52	1.86
[*L-Phe*, α-CD]	2.48	1.39	1.74	1.51	1.49	0.97
[*L-Phe*, β-CD]	3.13	0.63	0.76	0.91	0.81	0.53

[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.

solutions, where the solution viscosities were controlled in a wide range (from 1.51 to 4.91 cp) by adding various amounts of D-glucose[6]. In this situation, specific interactions between solute L-phenylalanine, added D-glucose, and solvent water are not expected, and so the macroscopic viscosity can be related to the observed T_1 changes. The same NT_1–viscosity relation also held for a case of anisotropic reorientation[10,11].

We have used the $NT_1\eta$ value to investigate the effect of complex formation on the molecular motion of the guest molecule. The $NT_1\eta$ values are shown in Table II with the values of the solution viscosity η. It is noteworthy that the $NT_1\eta$ values for L-tyrosine in the free state are significantly smaller than those for L-phenylalanine, in spite of the resemblance of their molecular volume V_m. The molecular volumes of L-tyrosine and L-phenylalanine in alkaline solution, calculated by the atomic increments method based on the van der Waals radii of the constituent atoms[12], are ~ 157 and ~ 153 Å^3, respectively. The smaller $NT_1\eta$ values for L-tyrosine are attributable to the increase in apparent molecular volume caused by solvation of the ionised hydroxyl group.

The $NT_1\eta$ values for aromatic carbons C_δ and C_ε of L-tyrosine and L-phenylalanine in the free state are larger than those of C_ε and C_ζ. These results indicate the existence of rapid, internal rotation of the aromatic ring, since an internal rotation that is faster than the overall molecular motion increases the NT_1 value[6,13]. The $NT_1\eta$ values for L-phenylalanine also show the existence of internal rotation of the phenyl ring even in the complexed state.

As can be seen from the data in Table II, all of the $NT_1\eta$ values show a decrease, to greater or lesser extents, on the addition of cycloamylose. According to theoretical predictions, a decrease in $NT_1\eta$ value corresponds to an increase in molecular volume. For cycloamylose–L-tyrosine and –L-phenylalanine complexes, the greater the dynamic coupling between them, 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 III

VALUES OF $NT_1\eta$[a] RATIOS FOR THE COMPLEXED AND FREE STATES OF L-TYROSINE AND L-PHENYLALANINE

Compounds	$(NT_1\eta)_{complex}/(NT_1\eta)_{free}$				
	α	β	δ	ε	ζ
[*L-Tyr*, α-CD]	0.99	0.68	0.95	0.90	
[*L-Tyr*, β-CD]	0.47	0.39	0.35	0.30	
[*L-Phe*, α-CD]	0.73	0.87	0.59	0.59	0.52
[*L-Phe*, β-CD]	0.33	0.38	0.35	0.32	0.29

[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 I.

extent of the decrease in $NT_1\eta$ may be used as a measure of the strength of dynamic coupling of L-tyrosine and L-phenylalanine guests with host cycloamyloses.

In Table III, the ratios of $NT_1\eta$ values for the complexed and free states of the guests are shown. It is noticeable that the aromatic-ring carbons of L-tyrosine in the β-CD–L-tyrosine system and of L-phenylalanine in α- and β-CD–L-phenylalanine systems show larger decreases in the $NT_1\eta$ values than those for other carbons in the same systems, indicating a larger slowing-down of the internal rotation of the aromatic ring than that of the other part or of the overall molecular reorientation. The results clearly show the formation of inclusion complexes of the guest L-tyrosine and L-phenylalanine with the host cycloamyloses by insertion of the aromatic ring into the cavity of cycloamylose.

The $NT_1\eta$ values of the aromatic carbons of both guests suffer a larger reduction on complexing with β-CD than with α-CD. These findings reflect the effect of cavity size on complex formation. According to the space-filling (CPK) model, β-CD has the best cavity for deep and tight inclusion of the phenyl ring, whereas the cavity of α-CD is too small for deep inclusion of the phenyl ring. As a result, the guest phenyl-ring is more strongly fixed in the cavity of β-CD and rather loosely in the cavity of α-CD.

Several mechanisms have been proposed for the formation of cycloamylose inclusion-complexes[2,7,14–16], but the forces leading to complexation are still unclear and a matter of speculation[2,7]. Among the proposed mechanisms, hydrophobic interaction seems to be the most probable as the driving force for the systems investigated here. In this case, the presence of additional interactions will alter the strength of dynamic coupling between the host cycloamylose and the guest amino acid. There are no appreciable differences in the extent of the decrease in $NT_1\eta$ for L-tyrosine and L-phenylalanine when complexed with β-CD. Thus, the ionised hydroxyl group of L-tyrosine has no influence on the dynamics of complex formation.

An interesting exception is the result for the α-CD–L-tyrosine system. The $NT_1\eta$ values for aromatic and α carbons of L-tyrosine decrease slightly or remain unchanged when it is complexed with α-CD, whereas there is a large decrease for the β-carbon. These results contrast with those for the α-CD–L-phenylalanine system and indicate that L-tyrosine cannot form an inclusion complex with α-CD, as also suggested by the small displacements of ^{13}C-chemical shift. A consideration of the concept of hydrophobic interaction, with the aid of a space-filling model, also accounts for these results. As indicated above, the cavity size of α-CD is too small for deep inclusion of the phenyl ring. If the aromatic ring of L-tyrosine is included in the cavity of α-CD in the same fashion as for L-phenylalanine, the ionised hydroxyl group must reside within the hydrophobic cavity. This situation is energetically disadvantageous as compared to the free state in which the hydroxyl anion is solvated by surrounding solvent molecules. For the β-CD–L-tyrosine system, the hydroxyl anion on the included aromatic ring is situated at the opposite end of the cavity and is exposed to the medium or to the primary hydroxyl groups of β-CD. The relatively

large decrease in the $NT_1\eta$ value for C_β in the α-CD–L-tyrosine system suggests the occurrence of interactions other than the inclusion type with α-CD.

The inclusion complexes between cycloamyloses and the aromatic amino acids investigated here show some characteristics of an enzyme–substrate complex[4]. The host cycloamyloses have a hydrophobic cavity leading to specific binding of the guest. The hydrophobic interaction seems to be the most probable as the driving force for the formation of a stable complex, and an additional hydrophilic group situated within the hydrophobic cavity makes the complex unstable. The geometrical matching between the host cavity and the guest inserting-group is one of the important factors determining the strong, dynamic coupling between them. The characterisation of complex formation by cycloamylose by molecular dynamics should yield information useful for the application of cycloamyloses in various fields.

Finally, reference should be made to the validity of the assignment of η in eq. *3* as the solution viscosity (macroviscosity). In some cases[17], the macroviscosity of a solution cannot be related to ^{13}C-T_1. For these cases, the microviscosity, as defined by the various local motions and local molecular interactions, should be used as the η value. Thus, alternative explanations are possible for the cause of the decrease in $NT_1\eta$ for the guest induced by complex formation. The macroviscosity may be used as the η value and the changes in $NT_1\eta$ values attributed to those in apparent molecular volume. Alternatively, the molecular volume may be regarded as constant and the changes in $NT_1\eta$ attributed to those in microviscosity. Our discussions are based on the first alternative, because we have no means of measuring the microviscosity except by the n.m.r. method[17]. Since the NT_1 values of L-phenylalanine in the free state were inversely proportional to the macroviscosity, as expected from the diffusion model, we can safely discuss the complexation effects on the molecular dynamics based on the first alternative.

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