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An Inclusion Complex of β-Cyclodextrin-L-Phenylalanine: ^{1}H NMR and Molecular Docking Studies

Pornthep Sompornpisut*, Nutsima Deechalao, Jitraporn Vongsvivut
Department of Chemistry, Faculty of Science, Chulalongkorn University,
Phayathai Road, Bangkok 10330, Thailand.
* Corresponding author, E-mail: spornthe@pioneer.netserv.chula.ac.th

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ABSTRACT An inclusion host-guest complex between β-cyclodextrin (β-CD) and L-phenylalanine (L-Phe) was investigated using ^{1}H nuclear magnetic resonance spectroscopy and molecular docking techniques. ^{1}H chemical shift changes of β-CD were used to calculate the stability constant (K_{stb}) of the complex. On the basis of the Hildebrand-Benesi method, the K_{stb} of the 1:1 complex in D_2O solution at 300 K, pD 7.6 was of 25.5 M^{-1}, implying a fast intermolecular exchange rate process. Interestingly, docking simulation indicates the toroidal space can be occupied by L-Phe with two favorable arrangements. For the predicted model with the higher probability score, the L-Phe aromatic ring is facing to the secondary hydroxyl groups of β-CD. Results from NMR and docking simulation are in good agreement with the x-ray structures of β-CD/L-phenylalanine derivatives.

KEYWORDS: β-cyclodextrin, Inclusion complex, ^{1}H-NMR; Molecular docking.

INTRODUCTION

β-cyclodextrins (β-CD) are cyclic oligosaccharides (Fig 1A) composed of seven (1→4)-linked α-D-glucopyranosyl units (Fig 1B). β-CD has a hydrophobic toroidal hollow truncated shape (Fig 1C), with a diameter of 6.0-6.5Å.[1-3] β-CD is widely used as a host-molecule, which can encapsulate/release various guest molecules. The stability of inclusion complexes depends molecular size and shape complementation but also the external environment and its conditions.[4] In addition, hydrophilic surface contacts of the primary and secondary hydroxyl groups located around the upper and lower perimeter of the β-CD molecule enhance its solubility in polar solvents. The dual properties of hydrophobic-interior and hydrophilic-exterior, such as seen in β-CD, are ideal for molecular carriers for use in several industrial and pharmaceutical applications.

^{1}H NMR spectroscopy is one of the most useful techniques for investigating the stability and stoichiometry of complexes, including host-guest systems.[3] The NMR technique provides direct and detailed observation of individual nuclei relevant to the structure and dynamics of the system. A previous study of an inclusion of the racemic mixture DL-phenylalanine (DL-Phe) with β-CD reported that the stability constant (K_{stb}) was 131 M^{-1} [5]. The fact is that the host molecule is known to be a chirality-selective molecule. For this reason, the binding constant of both enantiomeric forms might not be equivalent. The previously reported K_{stb} value was the NMR average of a dynamic equilibrium of two intermolecular exchange processes of D-Phe/β-CD and L-Phe/β-CD inclusion complexes. However, it is difficult to demonstrate our hypothesis of differential complex stability from that data set because it is impossible to quantitatively determine each inclusion complex from the mixture.

Here, we report a ^{1}H-NMR study of the complex between β-cyclodextrin and L-phenylalanine in aqueous solution. In this study, we are interested in determining the K_{stb} value of the inclusion of β-CD with L-Phe. In addition, a prediction of the β-CD/L-Phe inclusion structure was carried out using molecular docking techniques. L-Phe is a good choice for NMR investigation for understanding the basic for molecular recognition of amino acids and peptides by β-CD, because of the magnetically anisotropic shielding effects from the ring current. In addition, the L-stereoisomeric form is found in all naturally occurring amino acids in proteins. Such NMR and docking techniques should provide insights into the details of molecular interactions of amino acids with β-CD.

Materials and Methods

Experimental section

Materials

L-Phenylalanine (L-Phe: analytical grade) obtained from Calbiochem (La Jolla, CA, USA), β-cyclodextrin (β-CD: water content 10-13 %) acquired from Fluka (Buchs, Switzerland), and D_2O (deuterium content 99.9 %) purchased from Cambridge Isotope Laboratory (Andover, MA) were used for all sample preparations.

^{1}H NMR spectroscopy

Stock solutions of 0.010 M L-Phe and 0.010 M β-CD were made up in D_2O. The L-Phe and β-CD stock solutions were subsequently mixed as required. NMR spectra were recorded for a series of mixtures. Molar ratios of [L-Phe]/[β-CD] were varied from 0:1 to 10:1. 1D ^{1}H NMR spectra were acquired on Bruker DRX-400 operating at 400.13 MHz proton frequency at room temperature. The chemical shift at 4.75 ppm (1900.62 Hz) due to residual solvents (H_2O and HDO) was used as internal reference. The NMR signals are reported in Hertz throughout this report. The programs XWINNMR (Bruker Analytik GmbH, Germany) and MestRe-C 1.3.0 were used for NMR data processing.[6]

Computational details

Starting coordinates

The prediction of the inclusion complex between β-CD and L-Phe was begun from the construction of the three dimensional structures of the host and guest molecules. Atomic coordinates of β-CD were

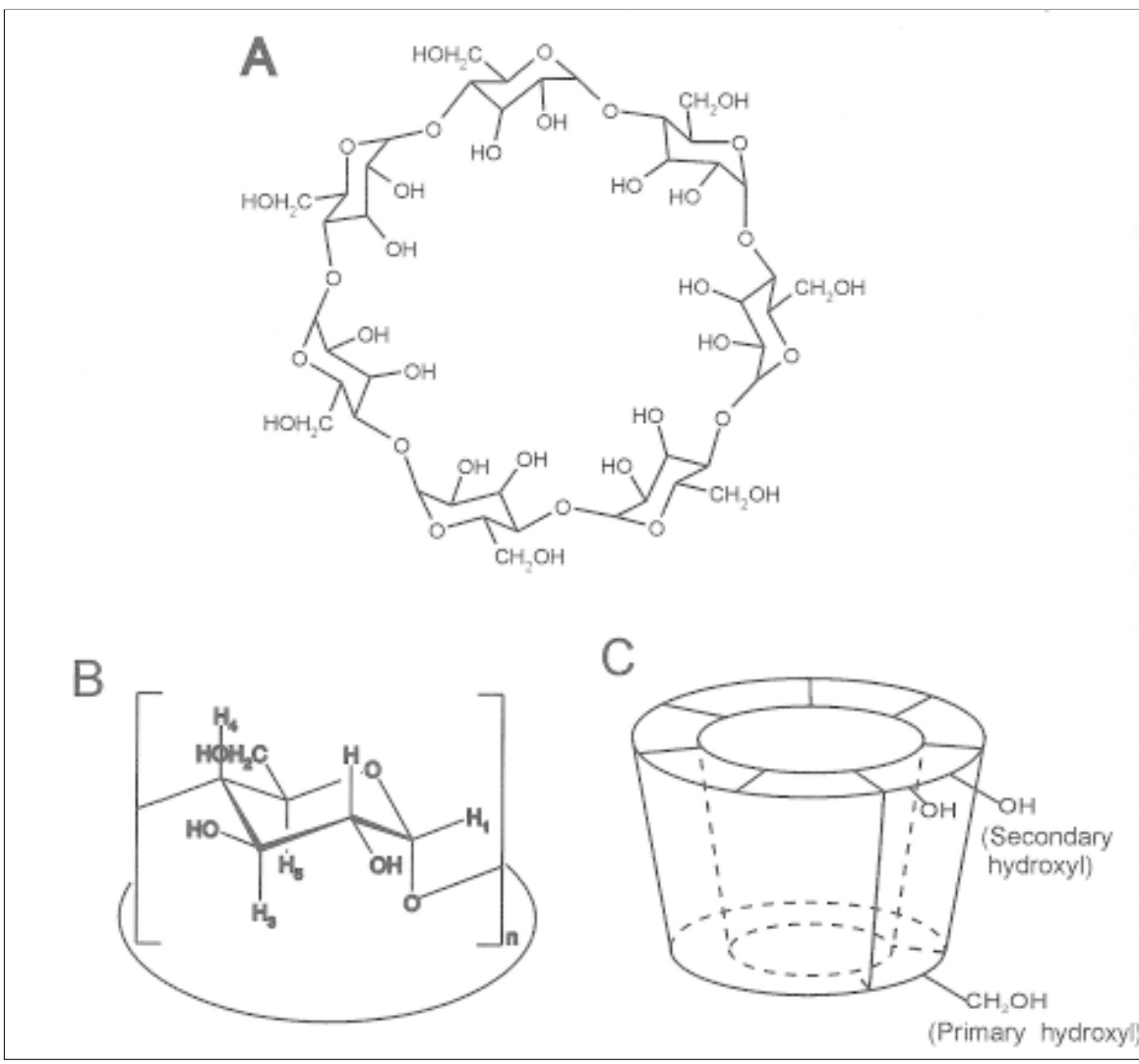

Fig 1. Schematic representation of β-cyclodextrin, (A) a cyclic oligosaccharide (top view) (B) the 1→4-linked α-D-glucopyranosyl unit and (C) a truncated torus shape (side view).

taken from X-ray crystallographic database. Hydrogen atoms were added to the x-ray structure and then structurally optimized employing geometric constraints for all heavy atoms. The partial atomic charges of β-CD were obtained from the fitting of the electrostatic potentials with the electron density for each atom using RESP module available in the program AMBER.[7] The electron density was calculated based on Mulliken population analysis[8] using the program Gaussian.[9] The L-Phe conformation was constructed and subsequently subjected to energy minimization using the semi-empirical AM1 method. Since the energy evaluation in the docking program is based on empirical molecular mechanic force fields, standard atomic charges of the L-Phe molecule were taken from Cornell's AMBER force field.[7]

Docking setup

A docking simulation were carried out using the program Autodock 2.4.[10] To search for favorable interaction energies between the host and guest molecules, grids were laid over a three-dimensional cubic box (25 x 25 x 25 $Å^3$) where the host molecule occupied the center of the box. The probe atoms (C, N, O, H) were used in order to pre-calculate the potential energy for each pair representing an interaction between the probe atom and the host atom being considered. Then all pre-calculated potential energies were stored in the database as the potential energy grid maps. At every point the interaction energy was derived from 12,6-Lennard-Jones potentials for van der Waals interactions and Coulomb potentials for electrostatic interactions. All parameters of energy calculations were taken from Cornell's force field.[7]

To search for suitable interaction sites, the guest molecule, L-Phe, was randomly moved within the 3D space around the fixed host molecule. Any movement can be regarded as a small atomic displacement consisting of translations and rotations of the guest molecule. To allow the guest molecule more flexibility, the torsion angles of the rotatable bonds are also varied. Each displacement can be referred to as one step of conformational search. Every single step was evaluated by calculating interaction energy, which is subsequently taken into consideration in the Monte Carlo decision routine. A cycle was reached when a maximum of 30,000 accepted or rejected steps took place. A single run is composed of 100 cycles. For statistical analysis, fifty independent runs have been examined, generating fifty models of inclusion β-CD/L-Phe structures.

Analysis of the results

We used a probability score, p, introduced previously by Sotrifer et al[11] to evaluate the results of docking simulations. This parameter systematically describes how well the predicted structures behave statistically and energetically. Therefore, the calculation of the probability score includes the combination of the occurring frequency (H_n) and the corresponding interaction energy (E_n) as follows:

$$p = \frac{H_n}{N} \exp\left(-\frac{E_n - E_1}{5.94} \right) \qquad (1)$$

where H_n refers to the total number of docking models in which L-Phe adopted similar orientations. N is the total number of runs. E_n is the interaction energy of the n^{th} predicted model and E_l is the lowest energy of the runs. The highest score corresponds to the most favorable model.

Autodock were performed on a Pentium pro computer. The models were visualized using the program Rasmol 2.6[12] and Weblab Viewer (Accelrys Inc, San Diego, CA).

Results and discussion

^{1}H chemical shift changes as a function of L-Phe concentration

The ^{1}H NMR signals belonging to the free forms of L-Phe and β-CD in aqueous solution have been previously assigned and the spectra obtained from the current experiments were identical to the literature.[3, 13-14] Therefore we have taken the resonance assignments for further study in the host-guest inclusion complex.

^{1}H NMR spectra of β-CD as a function of L-Phe concentration are shown in Figure 2. Here, we were able to observe shifted NMR signals for H3, H5 and H6 (Fig 2B, region c) while the H2 and H4 peaks remained unchanged (Fig 2B, region d). This indicates that the shifted peaks of the host protons are L-Phe dependent, being caused by the inclusion phenomena. Such changes suggested that protons at the 3rd, 5th and 6th positions of β-CD experience magnetic perturbation due to the guest molecules while those at the 2nd and 4th positions have no significant change in the chemical shift.

It should be noted that these observations are in good agreement with the recent crystal structure of β-CD and L-Phe derivatives.[15] The recent crystal structure reveals that the host-guest β-CD/L-Phe complex is a dimeric form. Each β-CD cavity of the dimer contains one molecule of L-Phe with slightly

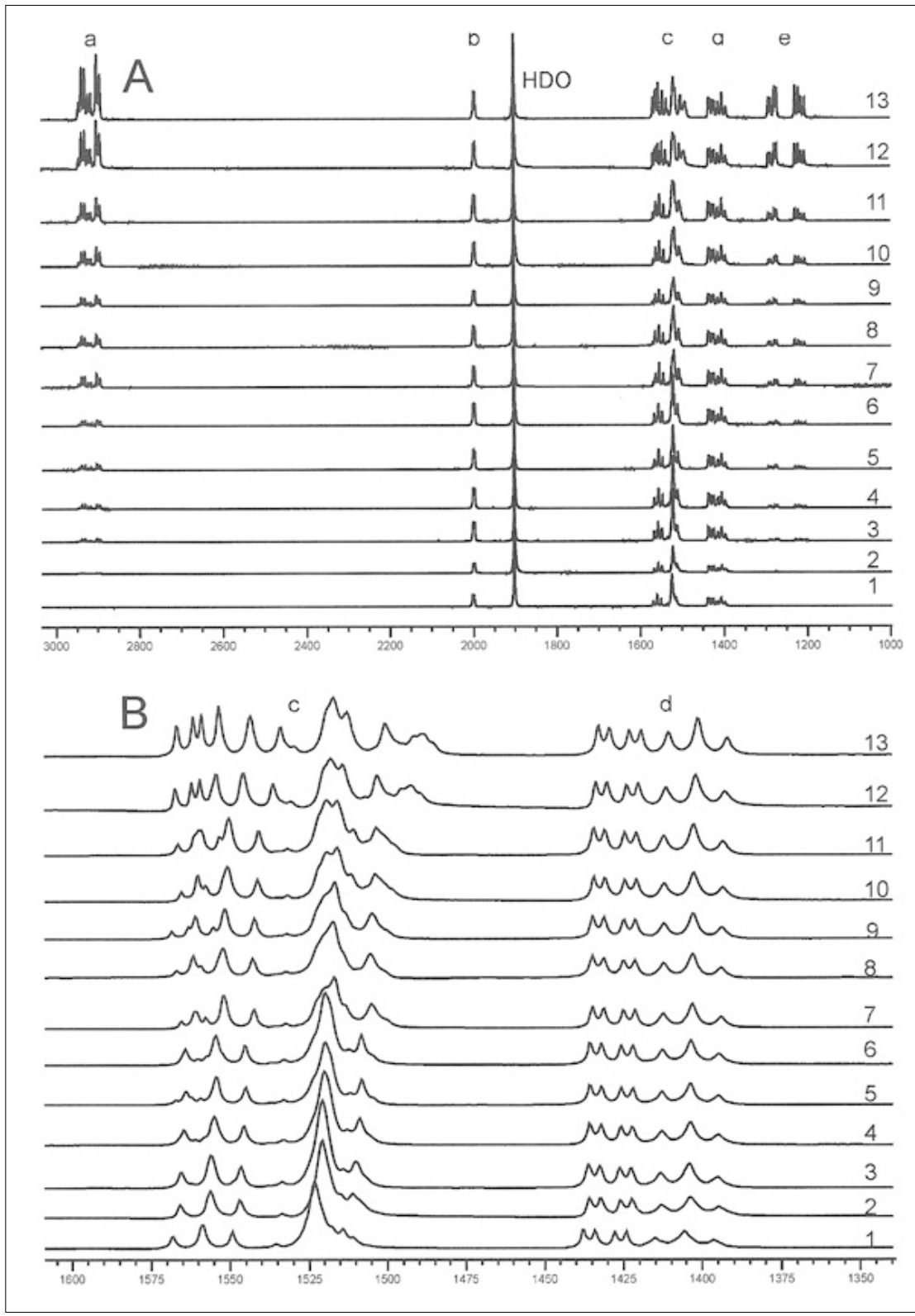

Fig 2. (A) ^{1}H NMR spectra as a function of molar ratios of β-CD/L-Phe ranging from 1:0 (#1) to 1:6.7 (#13). ^{1}H Peaks in each region (a-e) are as follows: aromatic L-Phe (a), H1 (b), H3, H5, H6 and Hα (c), H2 and H4 (d), and Hβ (e). (B) An enlargement of selected regions demonstrating shifted peaks for H3, H5 and H6 protons (c) but H2 and H4 relatively unchanged (d). Protons are labeled according to the commonly used standard.

different orientation. Therefore, hydrophobic interactions between an aromatic ring of the amino acid and hydrogen atoms in the β-CD cavity should be present in the complex. Since the C-H bonds of positions 3, 5 and 6 of the glucopyranosyl unit point toward the β-CD cavity, shielding effects due to the aromatic ring current should influence the chemical shifts of the host protons. The upfield-shifted signals of H3 and H5 suggested that the majority of those protons should not be exactly in the plane of the ring current. In addition, those shifted peaks are undistinguishable between the bound and the free states of β-CD; implying a rapid intermolecular exchange rate relative to the NMR time scale. On the other hand, the H2 and H4 protons do not directly interact with L-Phe because they are exposed to bulk environments.

Determination of the stability constant

The ^{1}H NMR study of the inclusion complex between β-CD and the racemic phenylalanine previously demonstrated that the complex had a 1:1 stoichiometric ratio and a stability constant (K_{stb}) of 131 M^{-1} [5]. To extract such information, the differences of the resonance frequency of the H3 proton were measured from the NMR spectra (Fig 2) because the signal is well-resolved and considerably intense. A plot of the differences of H3 resonance frequency *versus* the ratio of the concentration between L-Phe and β-CD was estimated to give the molar ratio of 1:1 for the complex. Thus, we were able to calculate the stability constant, K_{stb}, of the inclusion complex using the Hildebrand-Benesi equation modified for NMR applications.[16]

$$\frac{[\text{Phe}]_0}{\Delta\nu_{\text{obs}}} = \frac{[\text{Phe}]_0 + [\beta-\text{CD}]_0}{\Delta\nu_{\text{cpx}}} + \frac{1}{K_{\text{stb}}\Delta\nu_{\text{cpx}}} \quad (2)$$

$$\Delta\nu_{\text{obs}} = \nu_{\text{obs}} - \nu_{\beta\text{-CD}} \quad (3)$$

$$\Delta\nu_{\text{cpx}} = \nu_{\text{cpx}} - \nu_{\beta\text{-CD}} \quad (4)$$

where K_{stb} is the stability constant. $[Phe]_0$ and $[\beta\text{-CD}]_0$ are initial concentrations of the guest and host, respectively. $\Delta\nu_{obs}$ is the difference of the resonance frequency between the complex (ν_{obs}) and the free component ($\nu_{\beta\text{-CD}}$) observed from the experiment; and $\Delta\nu_{cpx}$ is the difference of the resonance frequency between a pure sample of the inclusion complex (ν_{cpx}) and β-CD ($\nu_{\beta\text{-CD}}$). The slope of the plot of $[Phe]_0/\Delta\nu_{obs}$ against $[Phe]_0+[\beta\text{-CD}]_0$ is equal to $1/(\Delta\nu_{cpx})$, and the y-intercept is equal to $1/K_{stb}(\Delta\nu_{cpx})$, allowing an estimation of K_{stb}.

The linear fitted plot (Fig 3) leads to K_{stb} of 25.5 M^{-1}. This value is in the order of the fast chemical change rate. It should be noticed that K_{stb} is significantly different from that obtained from the β-CD/DL-Phe complex[5], implying different stability of the complex of the D- and L-forms. Such discrepancy suggested that, under the experimental conditions, β-CD molecules bind to D-Phe tighter than to L-Phe. An inspection of the crystal structures suggested the hydrogen bonding networks play an important role for the stability of the host-guest complex. The hydrogen bonding interactions involve β-CD, L-Phe and a number of water molecules.[16] We anticipate that such interactions should be different between the two enantiomeric forms, even though the crystal structure and NMR dataset of the β-CD/D-Phe complex are currently unavailable,

The inclusion structure via molecular docking

From a total of fifty models predicted from docking simulations, forty-seven out of the fifty show the L-Phe molecule can be accommodated in the β-CD cavity. The convergence of the conformational search indicates a reliability of the results for extracting structural details. From the predicted models, the relative orientation of L-Phe molecule with respect to the β-CD cavity allows us to separate them into two distinct clusters (Fig 4). The first cluster constitutes of 24 out of 47 that arrange the aromatic side-chain against the secondary hydroxyl groups (Fig 4A) while the second cluster exhibited an opposite orientation (Fig 4B). In other words, the aromatic ring in the second cluster is closer to the

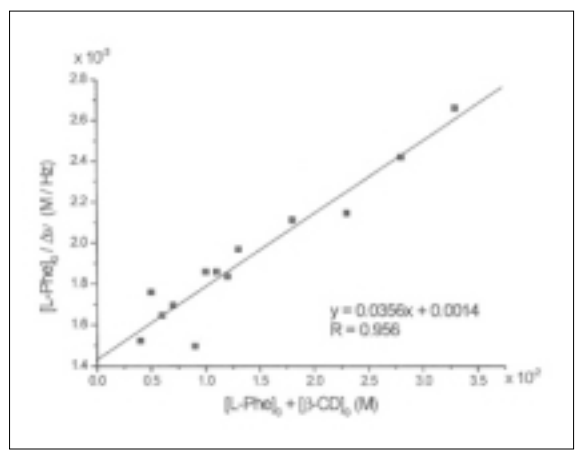

Fig 3. A linear fit curve (solid line) of experimental data of $[\text{L-Phe}]_0$ / Δν of the H3 protons of β-CD versus the total concentration.

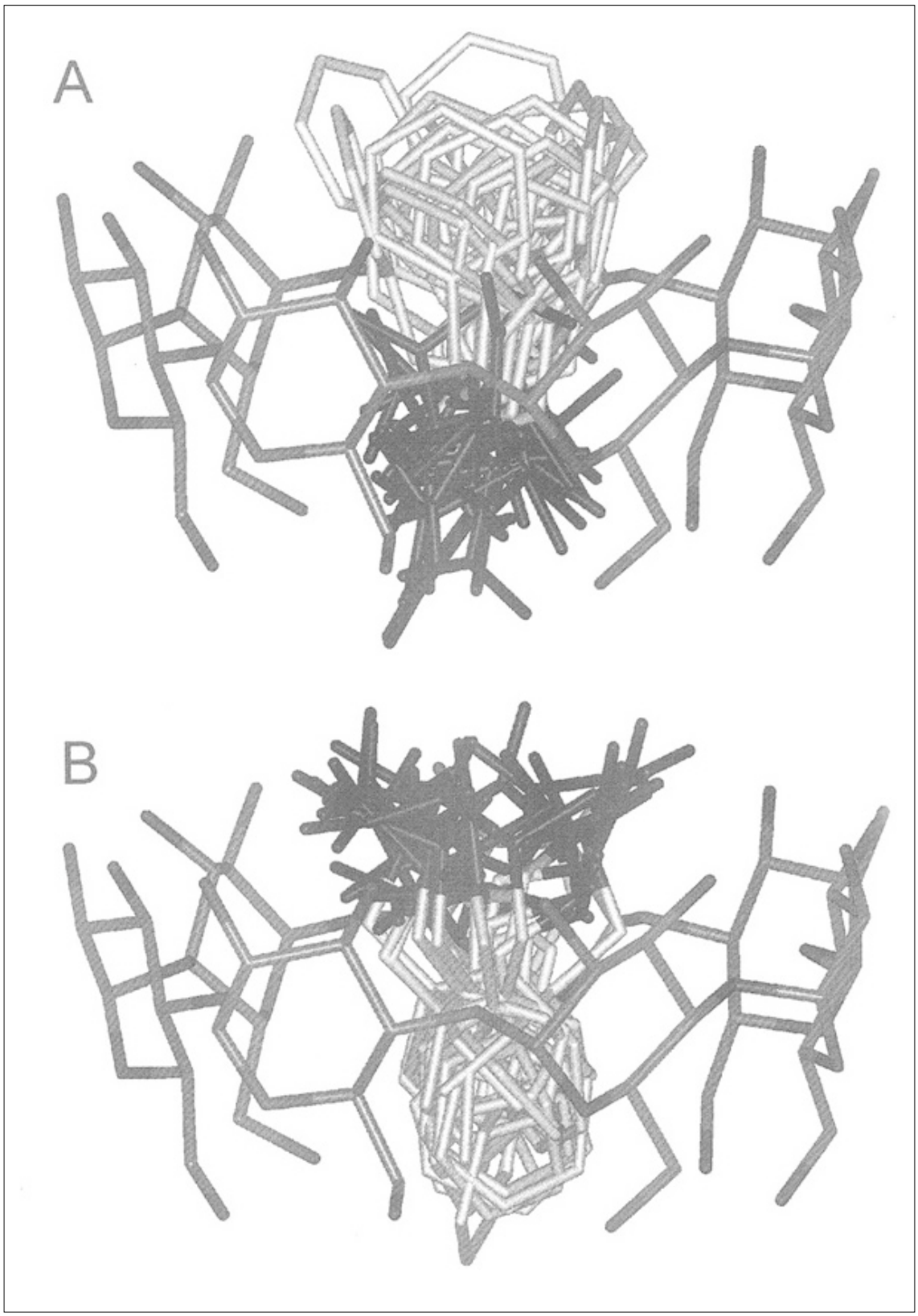

Fig 4. The predicted ensembles of the two possible arrangements of L-Phe in the β-CD cavity: (A) the aromatic sidechains of L-Phe near the outer mouth of β-CD with p=0.41; (B) opposite orientation relative to (A) with p =0.34.

primary OH groups than the secondary OH groups. For simplification, we shall call the first ensemble *Up* and the second ensemble *Down*. The calculated interaction energy of the *Up* ensemble was of –32.89 kcal/mol, which is insignificantly different from that of the *Down* ensemble (-31.96 kcal/mol). In addition, the number of the predicted models of both ensembles is nearly equivalent. An interpretation is that both arrange-ments may exist in the system or it is just an insufficient evaluation procedure. However, this has left it difficult to justify the possible binding mode of this system without further analysis.

The use of a scoring function (Eq 1) showed that the probability of L-Phe in the *Up* position (p = 0.41) was slightly greater than that of the *Down* position (p = 0.34). It should be noted that the arrangement of the phenyl sidechain in the x-ray structure is consistent with the ensemble with the higher probability score.

Nevertheless, the predicted results for the *Down* orientation should not be neglected because the statistic and energetic values are almost equal. There are two explanations for such results. As mentioned above, a first possible explanation is that the host cavity is too wide for specific orientations of L-Phe. The complex requires the assistance of some additional molecule(s) beyond the host and guest. This idea is supported by the x-ray structure, which showed that hydrogen bonding interactions involved the OH groups of β-CD, the carboxyl and amide groups of L-Phe and water molecules. Therefore the helper-water molecules stabilize the complex by hydrogen bonding. As a result, the two-body docking approach fails to provide the best fit of this case.

The second explanation is that under the crystallographic conditions, the structure with the lowest energy is preferable as the system gradually moves toward the minimum energy. This is not the case for the system undergoing thermodynamics equilibrium in solution. Thus two arrangements of L-Phe from this prediction may be possible.

Comparison between NMR and Docking techniques

NMR provides good descriptions regarding structural information and dynamic characteristics of the inclusion phenomena. The NMR technique used here is simple, yet powerful for investigating structural and dynamic properties of the host-guest complex. In this study, we have demonstrated that the β-CD/L-Phe system undergoes fast intermolecular exchange(K_{stb} ~25.5 M^{-1}). The shielding effect on ^1H chemical shift can be used to demonstrate the influence of the host-guest complex. As described previously, predicted models indicate L-Phe included in the β-CD cavity. The models are supported by the shifted signals for H3, H5 and H6 and the unchanged signals for the H2 and H4 protons of β-CD. In addition, this study shows a good agreement between the x-ray, NMR and docking models.

Although the docking approach does not provide very precise results, its predictive ability has been substantially demonstrated. It should be mentioned that the search algorithm used by the docking method is purely random and no distance restraints are employed in the calculations. Docking results show that the β-CD void pocket is viable for L-Phe and orientations of the guest molecule are considerably restricted. The search predicted only two most probable arrangements. In other words, error due to the prediction uncertainty was substantially reduced. This makes the use of the prediction results more meaningful for correlating with the experimental data.

Although the current NMR data were not able to confirm if the two predicted arrangements of L-Phe in the β-CD hydrophobic pocket do really exist. At the point, it could be due to a failure of the docking method but we have left this for future investigations.

Conclusions

We have carried out a simple but powerful ^1H NMR technique for characterizing the β-CD/L-Phe complex. The stability constant of an inclusion complex between β-CD/L-Phe indicates a fast chemical exchange rate relative to NMR time scale and is lower than that of β-CD/DL-Phe. The prediction of the host-guest complex model suggested that the cavity can be occupied by L-Phe with two possible arrangements, one of which agrees with the x-ray structure. The compliment between NMR and docking data is an attractive choice for investigating inclusion systems. NMR provides structural and dynamic properties of the inclusion phenomena while the docking method serves as supporting information.

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