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Chiral analyses of peptides by ion/molecule reactions

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Abstract

Protonated complexes comprised of guest peptides with permethylated hosts (β-cyclodextrin and maltoheptaose) were produced in the gas phase by electrospray ionization and reacted with a gaseous amine in a Fourier transform ion cyclotron resonance mass spectrometer. An exchange reaction was observed whereby the guest peptide was replaced by the gaseous alkyl amine. The exchange reaction was enantioselective and was used to determine enantiomeric excess in mixtures of enantiomers. The nature of the recognition is probed by the selection of the peptides and the use of molecular dynamics calculations.

Keywords: Chiral analyses; Peptides; Ion/molecule reactions

1. Introduction

A number of physical methods are useful for the differentiation of enantiomers. However, separations science offers the most practical methods for analytical applications. A general approach employs the creation of host–guest complexes. The relative rates of formation of the diastereoisomeric complexes vary under equilibrium conditions allowing the guest (analyte) compounds to be monitored according to their retention times as monitored by gas chromatography (GC) [1–5], high-performance liquid chromatography (HPLC) [6–9], or capillary eletrophoresis (CE) [6,10–13]. "Inclusion" or "host–guest" complexes capable of establishing non-covalent interactions with suitably sized analyte molecules hosted into the cavity are molecular recognition expressions often utilized in chiral studies. In general these interactions involve multiple non-covalent bonds between a large, geometrically concave molecule (host) and a smaller analyte molecule (guest). Geometrical requirements for this fit are essential. A number of chiral recognition models have been proposed to account for optical resolution by GC and LC, and they are most often based on the "three-point interaction" theory [14–17] first proposed by Easson and Stedman [18] and then Ogston [19]. According to this postulate, in order to obtain chiral discrimination, a minimum of three simultaneous, spatially significant contacts must exist. This rule is used in an uncritical way to rationalize chromatography experiments. The three contact points do not necessarily have to be points of molecular attachment. Repulsion or steric interactions can also facilitate molecular discrimination. The substantial complexity of the solution processes and the need to simplify the interaction conditions is one reason for studying host–guest complexation reactions in the gas phase.

The use of host–guest complexes in chiral mass spectrometry was widely studied by Sawada with ions produced by fast atom bombardment. The metastable decay rates of stereomeric host–guest complexes were found to vary [20–23]. Electrospray ionization on a quadrupole ion trap mass spectrometer was used to distinguish between three diastereomeric monosaccharides [24,25]. Dimeric product ions of Cu(II)-bound trimeric clusters formed by collisionally induced dissociation differ in enthalpy by only a few kilojoules per mole, thus, chiral recognition was achieved by Cooks and coworkers [26,27]. This method was used to determine enantiomeric excess (ee) with amino acids. It was subsequently used in the only other report of the determination of peptides [26]. In a similar vein, fragmentation of diastereomeric clusters between first-group metal ions and chiral aminophosphonic acids has been investigated by mass spectrometry [28]. Collision-induced dissociation (CID) of the clusters leads to fragmentation. The different spectral

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$$[HO{:}A + H]^+ + B \rightleftharpoons [HO{:}B + H]^+ + A$$

Scheme 1. Proposed mechanism for gas-phase enantioselective guest-exchange reaction.

features were correlated to the different stability of the diastereomeric complexes in the gas phase [28]. ESI-MS/MS was used for the chiral recognition of 19 common amino acids by employing collision-induced dissociation of complexes containing modified amino acids as chiral selectors [29,30]. More recently, the dissociation products and thresholds of neutral diastereomeric complexes of chiral compounds produced by supersonic expansion and probed by resonance-enhanced multiphoton ionization has been shown to be sensitive to the chirality of the constituent compounds [31,32].

Ion/molecule reactions of host–guest complexes in the gas phase have also been used for chiral recognition. The enantioselectivity is obtained from the different rates for enantiomers in the reactions. Fales and Wright reported one of the earliest studies on protonated dialkyltartrate dimers [33]. Other studies of the same or similar systems have followed, primarily by Nikolaev and coworkers [34–37]. Enantioselectivity in the dimerization of host–guest complexes have been reported by Dearden and coworkers [38,39]. Enantioselectivity has been observed in this laboratory in the gas-phase deprotonation reactions of gas-phase cytochrome *c* ions using chiral amines [40,41].

While the majority of these studies have focused on small molecules, particularly amino acids, there have been only one study of racemic mixtures of biopolymers such as peptides [26]. In the present study, we examined a small number of peptides with Phe and Val as the chiral residue with Gly spacers to understand the nature of enantioselectivity in the guest-exchange reaction summarized in Scheme 1. Enantiomeric analysis is achieved based on the differences in the reaction rates of gas-phase host–guest complexes. A diastereomeric complex $[HO{:}A + H]^+$ of an oligosaccharide host (HO) with a chiral analyte guest (A) reacts with an alkyl amine (base, B) to produce a product ($[HO{:}B + H]^+$) as illustrated in Scheme 1. The reaction is effectively a proton-transfer reaction mediated by a host molecule. This reaction has been studied extensively with amino acids [42,43]. The reaction has been employed in this laboratory to develop a method for enantiomeric determinations of amino acids [44,45] and pharmaceutical compounds [46]. A recent report by Speranza describes similar enantioselective gas-phase reactions with chiral resorcin [4]arene as host [47]. The same group also reports a unique enantioselective reaction involving diastereomeric acetate ions with tributylborate [48].

A series with Phe, Gly-Phe, and Gly-Gly-Phe (Fig. 1) was examined to observe the effect of lengthening the distance between the site of protonation (the N-terminus) and the chiral center (the Phe residue). Phe was chosen as it had the lowest enantioselectivity with permethylated β-cyclodextrin

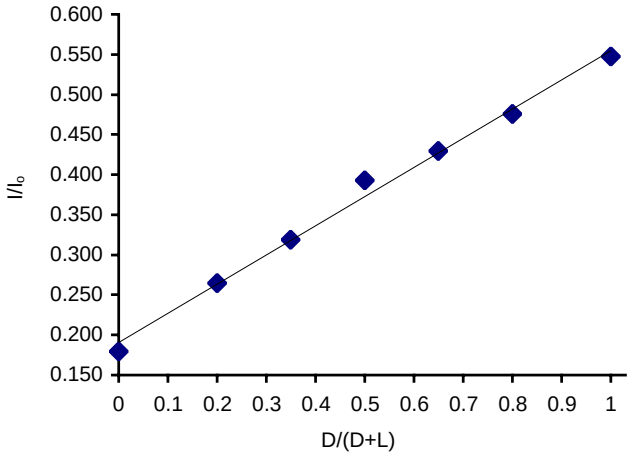

Fig. 1. Calibration curve for racemic mixtures of Gly-Phe. The complex $[\beta\text{-CD:Gly-Phe} + H]^+$ was reacted with ethylene diamine for a reaction time of 100 s.

(β-CD^m) but high selectivity with permethylated maltoheptaose (MP), the linear analog. For comparison Gly-Val and Phe-Gly were also included in the study. Val has one of the highest enantioselectivities with β-CD^m.

2. Experimental

2.1. Reagents

For the chiral exchange reactions, two different hosts were used: permethylated cyclodextrin, heptakis-(2,3,6-tri-*O*-methyl)-β-cyclodextrin, or β-CD, purchased from Sigma Chemical Co. (St. Louis, MO), and permethylated maltoheptaose, synthesized via the method given by Ciucanu and Kerek [49]. Amino acids were purchased from Sigma Chemical Co. and used without further purifications. Peptides used were Gly-Phe, Gly-Gly-Phe, Phe-Gly and Gly-Val. The L-forms were purchased from Sigma Chemical Co. The D-forms were synthesized for this study by Bachem, Inc. (Torrance, CA) and used with no further purification. The alkyl amines (B), *n*-propyl amine, ethylene diamine, and 1,3-diamino propane, were purchased from Aldrich Chemical Co. (Milwaukee, WI).

2.2. Procedure

All experiments were performed on a home-built electrospray FT-MS instrument described elsewhere [50,51]. The β-CD host solution was prepared by dissolving the methylated cyclodextrin in a water/methanol (50:50) solution, obtaining a concentration of 1×10^{-6} M. The concentration of maltoheptaose is approximately the same as that of CD. Analytes were dissolved in water/methanol (1×10^{-6} M). The electrospray solution was made by mixing a 100:1 ratio of analyte to host and was pumped at a rate of 8 μl/min. The electrospray needle was made of 50 μm fused silica

capillary. It was charged to approximately 1.8 kV to produce charged droplets that drifted downfield into the heated capillary. The resulting desolvated ions were transported into the analyzer cell of the FT-MS by a single stage quadrupole guide. The alkyl amine was purified in the vacuum manifold with several freeze-thaw cycles. It was then introduced into the analyzer chamber, allowing the pressure to become stable. Depending on the analyte, the alkyl amine pressure was maintained between 2 and 5×10^{-7} Torr. In the mass spectrum, the $[HO:A + H]^+$ is observed in large abundance along with β-CD coordinated to Na^+ and K^+—retained in the mass spectrum for use as internal standards—and some product $[HO:B + H]^+$ which is entirely ejected at the beginning of the experiment. Identical conditions were employed for the enantiomeric pairs.

2.3. Molecular modeling

The cyclodextrin and the protonated analyte structures were constructed and separately optimized fully using the Insight II builder module (Biosym, San Diego, CA) and the consistent valence force field (CVFF). The protonated oligosaccharide–analyte complexes were then formed by merging the respective oligosaccharide hosts and the analyte guests. Two sets of calculations were performed corresponding to two types of initial structures. In one set, the analytes were placed inside the cavity to produce initially inclusion complexes. In a second set, the analytes were placed on the outer wall of the host. In both cases, calculations of the complexes were started with fully optimized oligosaccharide host and amino acid structures. During the simulation, the structures of both the analytes and the hosts were allowed to fully optimize. The simulation was performed by heating the complexes to 600 K for 400 ps. At 8-ps intervals, a structure from the trajectory was captured and annealed in steps of 100 to 0 K. This resulted in 50 annealing simulations with a corresponding number of structures. All the structures within 5 kcal/mol of the lowest energy structure were examined and found to share the same structural features. Only the lowest energy structure of each enantiomer is presented.

3. Results

We chose a series of analytes with Phe and Val as the chiral residue with Gly spacers to determine the effect of lengthening distance between the site of protonation and the chiral center. Phe, Gly-Phe, Gly-Gly-Phe form a series of peptides with increasing distance between the site of protonation (N-terminus) and the center of chirality (Phe residue). For comparison, Phe-Gly was also examined. The reaction of the Phe-cyclodextrin complex $[\beta\text{-CD}^m\text{:Phe} + H]^+$ has been reported previously and was found anomalous compared to other amino acids. The selectivity ($S = k_l/k_d$, where k_l is the rate constants for the L-enantiomers) was relatively low ($S = 0.8$) [45]. For example, Val had relatively higher selectivity ($S = 2.1$). All peptides were examined with both permethylated β-cyclodextrin host (β-CD^m) and its linear analog permethylated maltoheptaose (MHP).

Proton transfer reactions yielding guest-exchange reactions (Scheme 1) occur when the gas-phase basicity of the amine is similar to or greater than the peptide. *N*-Propylamine (NPA) exchanged with Phe, however no appreciable reactions were observed with Gly-Phe or Gly-Gly-Phe. For the peptides, stronger bases such as ethylene diamine (EDA) and 1,3-diaminopropane (DAP) were necessary to affect reaction. Table 1 lists the rate constants for the L-enanntiomers of Phe, Gly-Phe and Gly-Gly-Phe reacting with NPA, EDA and DAP. As the peptide increases in size the reactivity decreases significantly even in situations where the basicity of the base is similar to that of the peptide. For example, the rate constant of the Gly-Gly-(L-)Phe complex reacting with DAP was relatively low at 8.8×10^{-14} cm^3/molecule s, although the basicities of the two are similar. L-Phe and NPA, for example, have similar basicities but the rate constant is significantly greater at 1.4×10^{-11} cm^3/molecule s. The greater steric hindrance associated with the larger peptides and the more intimate interaction between the host and the guest are the likely reasons for the greatly reduced rates.

A summary of the selectivities for the permethylated β-cyclodextrin (β-CD^m) host with the peptides is presented in Table 2. The most notable trend was the increase in selectivities between Phe ($S = 0.8$) and Gly-Phe (2.7). Selectivities were obtained from three sets of experiments with a deviation in the value of *S* being less than 10%. With Gly-Gly-Phe the selectivity decreases slightly but remains relatively high (2.0, 2.1). Selectivity appears to decrease with more basic alkyl amines but not to a large extent. The reaction of the Gly-Phe complex $[\beta\text{-CD}^m\text{:Gly-Phe} + H]^+$ becomes less selective with the more basic DAP (2.1) compared to the less basic EDA (2.7).

The enantioselectivity of the Phe-Gly complex appeared more similar to Phe than Gly-Phe. For Phe the D isomer was more reactive (albeit only slightly), but for Phe-Gly it

Table 1
Exchange rate constants of complexes with protonated peptides and permethylated β-cyclodextrin

Analyte	L-enantiomer ($\times 10^{-11}$ cm^3/molecule s)	D-enantiomer ($\times 10^{-11}$ cm^3/molecule s)	Alkylamine base
Phe	1.4	1.7	*n*-Propylamine
Gly-Phe	1.0	0.49	Ethylenediamine
Gly-Gly-Phe	0.0088	0.0044	1,3-Diaminopropane

Table 2
Reaction selectivities, $S = k_l/k_d$ for permethylated β-cyclodextrin as the host for selected peptides

Analytes		NPA (206 kcal/mol) [53]	EDA (215 kcal/mol) [53]	DAP (221 kcal/mol) [53]
Phe (217 kcal/mol)	*S*	0.80 [43]		
Gly-Phe (215 kcal/mol)[a]	*S*	NR	2.7	2.1
Gly-Gly-Phe (217 kcal/mol)[a]	*S*	NR		2.0
Phe-Gly	*S* (1/*S*)	NR		0.30 (3.3)
Val	*S*	3.1 [43]		
Gly-Val	*S*		0.87	

Values are average of three sets with deviation of less than 10%. The reactions were carried out with three alkyl amines as the base: NPA: *n*-propylamine, EDA: ethylenediamine, and DAP: 1,3-diaminopropane. Gas-phase basicities are listed.
[a] Values estimated based on Pro residue [54].

was three times more reactive ($S = 0.33$). This behavior contrasted greatly with Gly-Phe where the L-enantiomer was significantly more reactive.

The behavior of Val and Gly-Val is contrary to that of Phe and Gly-Phe. Rather than increasing, the value *S* for Gly-Val (0.87) decreases significantly compared to Val indicating that the D isomer is significantly more favored in the peptide than in the amino acid.

The use of permethylated maltoheptaose as host yielded high selectivities for the dipeptides (Table 3). The selectivity for the Phe containing group increases from Phe (4.6) to Gly-Phe (6.36) but decreases for Gly-Gly-Phe (1.7). The selectivity decreases from Phe to Phe-Gly (2.2) as was observed for the cyclic cyclodextrin hosts. For Val and Gly-Val, the selectivity increases significantly from 2.1 to 7.2.

3.1. Quantification and determination of enantiomeric excess

A calibration curve was constructed to determine whether the enantiomeric excess of peptides could be determined by mass spectrometry. Gly-Phe with β-CD as the host and ethylene diamine as the base, were chosen to construct calibration curves. Although selectivity is better with maltoheptaose as the host, β-CD was chosen for the practical reason that the exchange reaction is the fastest. To produce the calibration curve the relative peak heights of the reactants and products were used rather than the rate constants. This method has proved to be robust for the chiral analyses of amino acids and chiral drugs [46]. The procedure involves first choosing the proper conditions for the exchange reaction. Parameters includes the choice of alkyl amine, its pressure, and a reaction time that provides the shortest analysis time with sufficiently large differences in the relative intensities of the reactant and product complexes. For the standard mixtures, the enantiomeric composition was varied in this manner (L:D): 100:0, 80:20, 65:35, 50:50, 35:65, 20:80, 0:100. Seven points were used to create the curve for Gly-Phe in Fig. 1, although as little as two points were often found to be sufficient. The calibration curve shown in Fig. 1 has a coefficient of determination (r^2) of 0.987. Mass spectra for these mixtures were obtained at the chosen reaction time. From the mass spectra, a calibration curve was constructed. The plot has as the ordinate I/I_0, with *I* as product peak and I_0 the sum of the reactant peak intensity and product peak intensity. The abscissa is D/(D + L), the Gly-D-Phe enantiomer mole fraction. The quality of the calibration curve depends on the value *S* with the largest errors obtained for mixtures where *S* was close to 1. Mixtures of various "unknown" enantiomeric content were later analyzed. In Table 4, the enantiomeric excess results for four unknown mixtures are summarized.

Table 3
Reaction selectivities, $S = k_l/k_d$ for permethylated maltoheptaose as the host for selected peptides

Analytes		NPA	EDA	DAP
Phe	*S*	4.6 [45]		
Gly-Phe	*S*		6.4	
	k_l		0.066	
	k_d		0.010	
Gly-Gly-Phe	*S*			1.7
	k_l			0.026
	k_d			0.016
Phe-Gly	*S*			2.2
				4.6
				2.1
Val	*S*	2.1 [45]		
Gly-Val	*S*		7.24	
			4.0	
			0.55	

The reactions were carried out with three alkyl amines as the base: NPA: *n*-propylamine, EDA: ethylenediamine, and DAP: 1,3-diaminopropane. Typical rate constants are provided, *k* ($\times 10^{-11}$ cm^3/molecule s). The values for *S* are averages of three runs.

Table 4
$[\beta\text{-CD:Gly-Phe} + H]^+$ reacting with ethylene diamine

Experimental D/(D + L)	Calculated D/(D + L)
0.13	0.09
0.43	0.38
0.71	0.66
0.91	0.79

Reaction time: 100 s. Results show determination of the D-enantiomer composition in four unknown mixtures analyzed using a calibration curve. Avg. error 15%.

4. Discussion and theoretical results

The poor selectivity observed with Phe employing β-CD^m ($S = 0.8$) as the host has been discussed in detail previously [45]. The relatively rigid cavity of β-CD^m constrains the phenyl group, which in turn forces the ammonium and the carboxylic group to interact with the cyclodextrin rims in limited ways. While it has been shown that some rigidity is important for achieving high enantioselectivity, we found that too much rigidity worked to decrease selectivity. The restriction placed by the cavity on the guest limited greatly the differences in interactions between the L- and the D-enantiomers for Phe thereby yielding the low selectivity observed ($S = 0.80$) [42,45]. In this context, the increasing selectivity from Phe to Gly-Phe and Gly-Gly-Phe with β-CD^m appears anomalous. In all three, the N-terminus is the site of protonation [52].

Comparing the selectivity of Phe, Gly-Phe, and Gly-Gly-Phe is complicated by the differences in the alkyl amine bases. A single base could be used for comparing all three compounds but that would mean reacting all three with DAP. This reaction has been examined, but it happened too quickly for the amino acid and the dipeptide to yield significantly reduced selectivities. For comparative purposes, we chose instead to maximize the selectivity by using bases with gas-phase basicities that closely matched the peptides.

We performed molecular modeling calculations to shed some insight into the selectivity. The peptide protonated at the N-terminus was allowed to geometrically optimize both inside and outside the cavity. The results always yielded the inclusion complexes. The protonated terminal amine was found in all cases to prefer interaction with the narrow (C^6-OH) rim (Fig. 2). Lengthening the compound effectively pushes the Phe group out of the cavity to maintain the interaction between the protonated N-terminus with the narrow rim. With the Phe group outside of the cavity, the L- and D-forms are not as strongly constrained and interact with the cyclodextrin host in more unique ways resulting in possibly greater enantioselectivity. In other words, the restriction imposed by the cavity due to the inclusion of the phenyl group is reduced when the phenyl group is pushed out of the cavity onto the wide rim in Gly-Phe and Gly-Gly-Phe (Fig. 2). While this is a plausible explanation, we cannot rule out the effect of the differences in gas-phase basicities or steric interaction brought about by the different bases.

Selectivity was markedly improved when maltoheptaose (MHP) was used as the host, with $S = 4.6$ for Phe and $S = 6.4$ for Gly-Phe (Table 3). The molecular modeling structures suggest that maltoheptaose host wraps around the guest and solvates it to produce a quasi-inclusion complex (Fig. 3). The most prevalent low-energy structure was one where the host wraps around the guest in a helical fashion producing intimate interactions. The linear host's adjustable cavity apparently may the phenyl group to find unique orientation for both isomers that are well distinguishable from one another by the exchange reaction. Indeed, ammonium and carboxylic groups in the L- and D-isomers appear to have distinct interactions with the maltoheptaose (Fig. 3). To the incoming base acquiring the proton, the L-complex structure is significantly different from the D to yield large differences in the exchange rates as reflected by the large S value.

For the cyclodextrin host, it has been shown that enantioselectivity increases with the number of carbons for amino acids having alkyl side chains—Ala, Val, and Ile [45]. In the three-point interaction model, maximal differentiation is created when two attractive and one repulsive interaction occur. As the R group increases, the repulsive interaction increases and selectivity increases. But in the Gly-Val case, selectivity is severely diminished, $S = 0.87$. The peptide is apparently too bulky for the cavity of β-CD. The ammonium and the carboxylic acid groups yield attractive interactions with the rims, but the hydrophobic side chain is on the outside, creating no third repulsive interaction. Fig. 4 shows that the hydrophilic top rim of the CD interacts with the carboxylic group of Val. The only fully included part is the achiral Gly portion of the peptide containing the exchangeable proton. Chiral differentiation likely occurs when access to this proton is hindered by either the host's rim methoxyl groups or

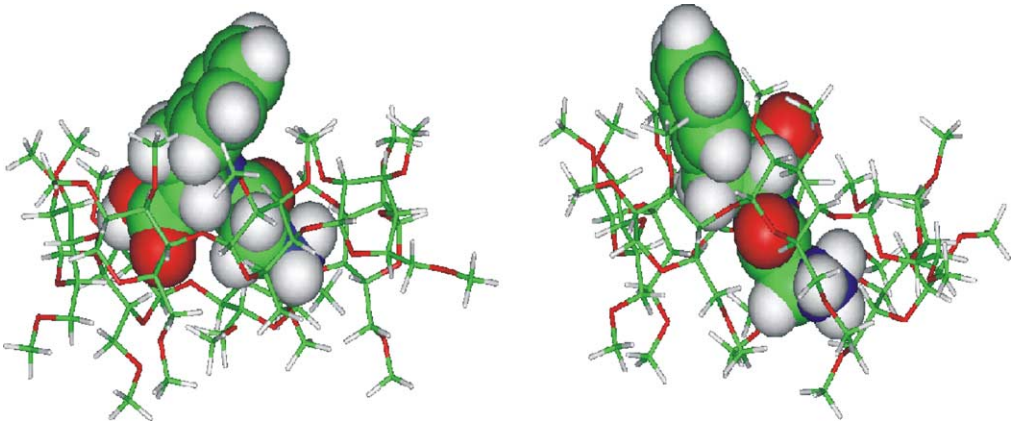

Fig. 2. Molecular dynamics result for Gly-L-Phe (left) and Gly-D-Phe (right) protonated in protonated cyclodextrin complex [β-CD^m:Gly-Phe + H]$^+$.

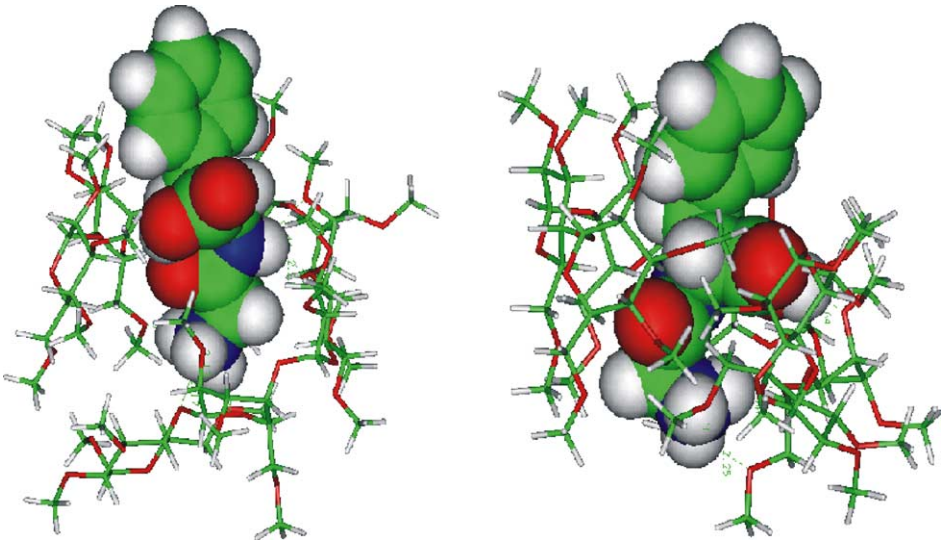

Fig. 3. Molecular modeling structure of protoanted Gly-L-Phe (left) and Gly-D-Phe (right) protonated in permethylated maltoheptaose [β-MHP^{m}:Gly-Phe + H]$^{+}$.

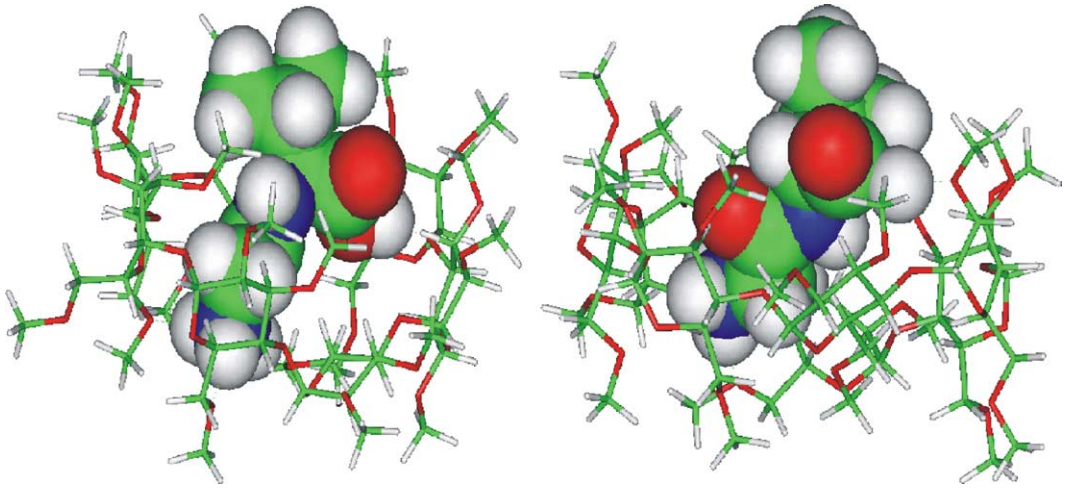

Fig. 4. Molecular modeling structure of [β-CD:Gly-L-Val+H]$^{+}$ (left) and [β-CD:Gly-D-Val+H]$^{+}$ (right).

the guest's alkyl side chains. To the incoming ethylene amine base, location of the exchangeable proton appears similar. Alternatively, rigidity of the host imposes conformational restraints on Gly-Val, impeding its two enantiomeric forms from achieving fits that are sufficiently different, much in the same way as the side chain of Phe decreases enantioselectivity. Exchange reaction for both L- and D-forms again proceeds at more similar rates. Enantioselectivity significantly increases when maltoheptaose is used as the host for Gly-Val. The selectivity value is $S = 7.2$. Flexibility of this host results in high selectivity for the alkyl side-chain analytes, again similar to the aromatic side-chain analytes.

5. Conclusion

The larger distances between the site of protonation and the center of chirality do not appear to diminish selectivity. However, compatibility between the size of the analyte and the size of the host is an important consideration in the selectivity. The host with a larger more flexible inner cavity (MHP) generally yielded the largest selectivities. Differences in chirality in peptides are readily discerned by the guest-exchange reactions. In the cases studied, the enantioselectivity of the peptides are significantly greater than the amino acids.

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