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The Binding of Free Oligopeptides to Cyclodextrins: The Role of the Tyrosine Group

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Abstract. The formation of α-cyclodextrin (α-CD) and β-cyclodextrin (β-CD) inclusion complexes with free tyrosine and the tyrosine residues within two free oligopeptides were investigated using steady-state fluorescence spectroscopy. The oligopeptides consist of five amino acids (pentapeptide) and the tyrosine residues are located at the *n*-termini. The two peptides used in this study have well-known biological functions and are known to bind selectively to specific cell receptors. Cyclodextrins were used to model this receptor–peptide (protein–ligand) interaction. Equilibrium binding constants and the enthalpy and entropy of binding were recovered. Molecular size of the tyrosine-containing species and pH (7.0 vs. 10.0) were found to have little affect on α-CD binding. However, tyrosine binding to β-CD was dependent on the size (free tyrosine vs. peptide), structure, and pentapeptide conformation.

Key words: Fluorescence, minimal peptides, tyrosine residues, cooperativity, cyclodextrins.

1. Introduction

Previous work from our laboratories has focused on producing specific cellular responses from minimal peptide sequences (MPS) covalently bound to fluoropolymer surfaces [1–3]. Minimal peptide sequences are the smallest sequential set of amino acids, from a larger protein unit, necessary to elicit a specific response. These MPS are known to promote specific cellular responses on binding to cell-surface receptors (integrins) [4, 5]. Adhesive proteins (e.g., fibronectin, vitronectin, fibrinogen) and extracelluar matrices (e.g., laminin, collagen, von Willebrand factor) have several binding sites for integrin–cell surface receptors [6]. Integrin receptor proteins are known to connect the cell interior to its exterior environment and allow the cell to 'see' and 'feel' its environment and respond accordingly. Integrin receptors can also recognize cell binding domains of three to five sequential amino acids within larger proteins.

An MPS of particular interest to us is a peptide fragment of laminin, tyr-ile-gly-ser-arg (YIGSR). YIGSR interacts specifically with the integrin receptors on certain neuronal cells [1, 3, 7]. The enkephalin YGGFL (tyr-gly-gly-phe-leu) serves as the body's naturally-formed opiate and they bind to brain receptor sites, where

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they induce analgesia and deaden pain sensations much like morphine and heroin [8].

The objective of this paper is to develop an understanding of how YGGFL and YIGSR bind and the role of the tyrosine residue in this binding process. To this end, we have studied the binding of free L-tyrosine, YIGSR and YGGFL to a simple protein surrogate, namely cyclodextrin [9–12].

The β-amyloid peptide [13] is the major component of senile plaque found in the brain of Alzheimer patients [14, 15], and the binding of β-cyclodextrin (β-CD) to aromatic amino acid residues on this peptide is known to decrease its *in vitro* neurotoxicity. In addition to its *in vitro* neurotoxic and neurotropic behavior, the β-amyloid peptide apparently functions as an adhesion molecule [16]. Camilleri et al. [13] attribute the decrease in *in vitro* neurotoxicity to binding between β-CD molecules and specific amino acid residues intrinsic to the β-amyloid peptide. β-Amyloid contains one tyrosine and three phenylalanine residues and electrospray ionization mass spectroscopy (ESI-MS) has shown that several β-CD molecules can bind to a single β-amyloid peptide. Additional work by Camilleri et al. [17] reported β-CD complexes forming with phenylalanine and tryptophan residues; tyrosine/β-CD complexes were not detected. This particular result may, of course, arise from the peculiarities of the ESI-MS ionization process because others have demonstrated that tyrosine/β-CD complexes can form [18–20].

It is well known that the B-chain of insulin forms dimers and oligomers under certain conditions [21]. Interestingly, the B-chain contains three phenylalanine and two tyrosine residues, and addition of β-CD inhibits insulin aggregation in solution [22]. Additional reports have appeared documenting the effects of cyclodextrins on other biological systems [23–30]; however, there are few published reports discussing the binding of tyrosine residues and the like to model hosts such as cyclodextrins [17–20]. This dearth of information is especially troubling, considering that previous work [13, 17, 22] suggests a critical role for tyrosine and other aromatic amino acid residues in several biologically relevant aggregation, association, and binding phenomena.

Matsuyama et al. [18] have used microcalorimetry to determine the equilibrium binding constant between tyrosine, phenylalanine, and tryptophan and α- and β-CD. Inoue and coworkers [20] studied the binding of phenylalanine and tyrosine to α- and β-CD using ^{1}H and ^{13}C-NMR. More recently, ESI-MS [17] and competitive spectrophotometry [19] were used to follow the binding of the aromatic amino acids with cyclodextrins. Reverse phase-high pressure liquid chromatography, using α- and β-CD as mobile phase modifiers, has also been used to improve the separation of aromatic amino acids, including tyrosine [31].

We report on the binding of free tyrosine and the tyrosine residues in free YIGSR and YGGFL to α- and β-CD in aqueous solution. The current work is to be *contrasted* with previous work [32, 33] on the behavior of YGGFL tethered covalently to cyclodextrin. In these previous offerings the aim was to use the peptide tether as a molecular recognition element and to deliver a host (sequestered within

the CD cavity) to a particular biological site. These previous studies [32, 33] also showed that the YGGFL tether did *not* form an intramolecular CD complex in D_2O or DMSO-d_6. Such is not the case in the current work with the free guest molecules, and we use changes in the steady-state tyrosine fluorescence to determine the equilibrium binding constant (K) and thermodynamic parameters (enthalpy and entropy). YIGSR contains one tyrosine residue at the n-terminus. YGGFL is slightly more complicated because it contains tyrosine *and* phenylalanine; individually both are reported to complex with cyclodextrins [18–20].

2. Experimental

Distilled deionized water was used to prepare all solutions and all materials were used as received. Cyclodextrins were from Sigma. Stock solutions (1.0×10^{-3} M) of the individual pentapeptides (YIGSR and YGGFL) and L-tyrosine (Sigma) were prepared in 0.01 M phosphate buffer, pH 7.0 or 0.01 M carbonate buffer, pH 10.0. All cyclodextrin stock solutions (α-CD, 0.100 M; β-CD, 0.014 M) were prepared in either 0.01 M phosphate buffer, pH 7.0 or 0.01 M carbonate buffer, pH 10.0 to maintain ionic strength. In the titration experiments, the cyclodextrin concentrations were systematically varied (0–0.100 M, α-CD; 0–0.014 M, β-CD) and the tyrosine, YIGSR, or YGGFL concentration was maintained at 5×10^{-6} M. All stock solutions were stored at 4 °C and used within two weeks of their initial preparation.

Steady-state fluorescence measurements were carried out using a SLM-Aminco model 48000 MHF spectrofluorometer [34, 35]. The sample temperature was maintained at the desired value (± 0.1 °C). All samples were allowed to equilibrate at each temperature for at least 30 min. Excitation was at 275 nm and emission was recorded from 290 to 400 nm. Excitation at 275 nm is selective for tyrosine residues, with no interference from phenylalanine or any other amino acid residues present in these particular oligopeptides. All spectra were blank corrected and the integrated area under the individual emission spectra (290–350 nm) as a function of added cyclodextrin was used as the measure of the fluorescence intensity.

3. Results

The tyrosine emission is a strong function of the physicochemical properties of its local environment. As a result, one can conveniently use changes in tyrosine fluorescence on titration with α- and β-CD as a means to determine the equilibrium binding constants (K) [36–39]. In this work, the Benesi–Hildebrand approach [37] was used to recover a set of 'seed' K values that were used within a nonlinear least-squares regression analysis of the actual fluorescence intensity data [38, 39]:

$$I = [I_0 + I_s K[\mathrm{CD}]_0]/[1 + K[\mathrm{CD}]_0] \quad (1)$$

where I is the observed fluorescence at a given CD concentration, I_0 is the intensity in the absence of added CD, I_S is the intensity from the fully formed complex, K

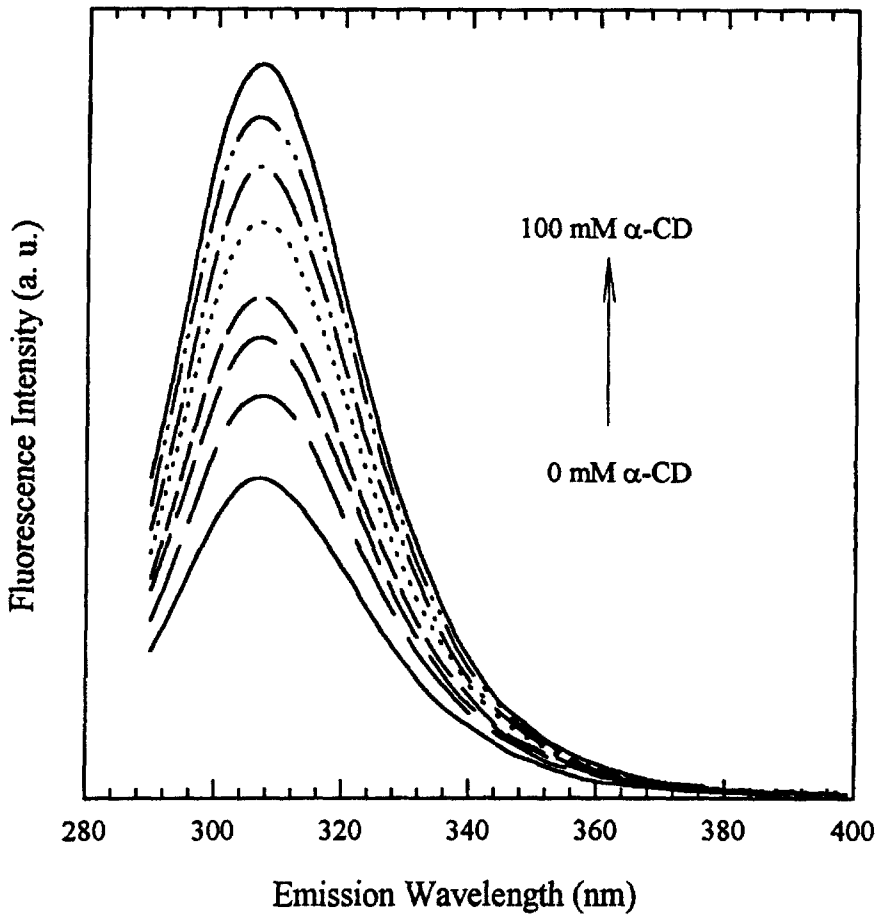

Figure 1. Steady-state fluorescence spectra of tyrosine (5×10^{-6} M) as a function of added α-CD.

is the equilibrium constant, and $[CD]_0$ is the analytical cyclodextrin concentration. Experimental intensity vs. CD data fit well to Equation 1 with $\chi^2 < 1.03$ in all cases, indicating 1 : 1 binding stoichiometry. The enthalpy (ΔH) and entropy (ΔS) of binding were calculated from a van't Hoff plot.

Figure 1 presents a typical series of emission spectra for 5×10^{-6} M tyrosine as a function of added α-CD. An enhancement in the tyrosine fluorescence on adding α-CD is clearly evident. This trend is also observed when titrating YIGSR and YGGFL with α- and β-CD (results not shown). Inclusion of tyrosine into the cyclodextrin cavity is thought to increase the fluorescence intensity by minimizing interactions that cause and/or promote nonradiative decay pathways from the excited state (quenching and solvent relaxation) [40]. In addition, the fluorescence quantum yield and excited-state lifetimes of phenol and several phenol derivatives are known to increase on binding to cyclodextrins [40].

Table I. Equilibrium binding constants for tyrosine, YIGSR, and YGGFL to α- and β-CD at 20 °C

	α-CD binding, K (M^{-1})		β-CD binding, K (M^{-1})
Guest	pH 7.0	pH 10.0	pH 7.0
L-tyrosine	27 ±3	23 ± 4	48 ± 5
YIGSR	20 ± 4	19 ± 4	224 ± 10
YGGFL	20 ± 3	20 ± 3	123 ± 15

Table I collects the equilibrium binding constants (20 °C) for tyrosine, YGGFL, and YIGSR with α- and β-CD at pH 7.0 and 10.0. Several aspects of these data merit special mention. First, free tyrosine and the tyrosine residues in free YGGFL and YIGSR have larger binding constants to β-CD relative to α-CD (a twofold increase for free tyrosine, tenfold for YIGSR, and sixfold for YGGFL). Second, changes in pH (from 7.0 to 10.0) have little, if any affect on the tyrosine binding to α-CD. Third, there is little difference in α-CD binding between free tyrosine and tyrosine at the n-terminus of either free pentapeptide. Fourth, tyrosine residue/β-CD binding is much greater for the free pentapeptides compared to free tyrosine. This may be a function of deeper and tighter penetration of the tyrosyl group within the β-CD cavity [20], with additional stabilization from hydrogen bonding between the oligopeptide backbone and secondary hydroxyl groups on the outer rim of the β-CD cavity. Finally, the K for YIGSR/β-CD is significantly greater than YGGFL/β-CD.

System pH does not affect the tyrosine/α-CD binding. Both pH values are significantly below the pK_a of the secondary hydroxyls on the outer rim of the cyclodextrin, which is 12 [9, 41]. Although the pK_a of tyrosine's phenolic hydroxyl function is 10.1, tyrosinate fluorescence, which is well-resolved from tyrosine fluorescence, was not observed in this work. It can thus be assumed that at pH 7.0 and 10.0, that tyrosine's phenolic group and the hydroxyls on the cyclodextrin remain protonated at pH 10. This suggests the CD cavity 'buffers' the phenolic residue and maintains the tyrosine in the –OH form.

The K for tyrosine and tyrosine residue complexation with α-CD is, for the most part, unaffected by the size of the tyrosine-containing molecule. This result suggests that inclusion of tyrosine's aromatic ring structure into the α-CD cavity is geometrically limited. Accessibility of the aromatic ring and secondary stabilizing interaction (e.g., hydrogen bonding), issues that might be associated with the pentapeptides, are apparently overridden and do not affect tyrosine residue binding to α-CD.

Temperature-dependent experiments (Tables II and III) were used to determine the enthalpy (ΔH) and entropy (ΔS) of binding for free tyrosine, YGGFL, and YIGSR to α- and β-CD (Table IV). The magnitude of the α-CD binding constants were generally a function of the temperature; pH had little influence. In the pres-

Table II. Temperature-dependent equilibrium binding constants for tyrosine, YIGSR, and YGGFL with α-CD

pH	Guest	Equilibrium constants, M^{-1} 283 K	293 K	303 K	313 K
7.0	Tyrosine	36 ± 5	27 ± 4	16 ± 4	13 ± 2
	YIGSR	33 ± 5	20 ± 4	15 ± 5	8 ± 3
	YGGFL	32 ± 4	20 ± 2	16 ± 2	11 ± 3
10.0	Tyrosine	33 ± 5	23 ± 5	19 ± 5	n.c.
	YIGSR	27 ± 2	19 ± 4	13 ± 3	n.c.
	YGGFL	25 ± 4	20 ± 1	14 ± 3	n.c.

n.c. Experiments not conducted.

Table III. Temperature-dependent equilibrium binding constants for tyrosine, YIGSR, and YGGFL with β-CD

Guest	Equilibrium constants, M^{-1} 283 K	293 K	303 K	313 K
Tyrosine	47 ± 6	48 ± 5	51 ± 9	33 ± 6
YIGSR	284 ± 10	224 ± 17	178 ± 16	151 ± 13
YGGFL	197 ± 21	123 ± 18	87 ± 10	43 ± 7

ence of β-CD, the recovered binding constants were found to be a function of guest molecule identity *and* temperature (Table III). The binding of free tyrosine to β-CD is about two-fold greater than with α-CD. However, the tyrosine/β-CD binding was essentially temperature invariant. YIGSR/ and YGGFL/β-CD equilibrium constants were considerably greater than the corresponding α-CD complexes and the tyrosine/β-CD complex. The recovered equilibrium constants were statistically different for the two pentapeptides with β-CD at the 95% confidence level. However, in both oligopeptides, tyrosine is the n-terminal amino acid; therefore, any differences in binding must arise from differences in tyrosine ring accessibility and/or the amino acid sequence of the two peptides.

Table IV collects the binding enthalpy and entropy for all the complexes studied. For α-CD binding, ΔH and ΔS values are all comparable for tyrosine, YGGFL, and YIGSR as a function of pH. This is not the case for β-CD binding, where the tyrosine, YGGFL, and YIGSR complexes exhibit significantly different ΔH and ΔS values. The K for the tyrosine/β-CD complex is essentially temperature independent; therefore, ΔS is close to zero. If the assumption that ΔS equals zero is made, ΔH equals $-(9 \pm 3)$ kJ/mol. This value is much lower than ΔH for tyrosine binding to α-CD and suggests that the near-zero change in order in the tyrosine/β-CD system compensates for the decrease in exothermicity.

Table IV. Binding enthalpy and entropy for α- and β-CD binding with tyrosine, YIGSR, and YGGFL

	α-CD, pH 7.0		α-CD, pH 10.0		β-CD, pH 7.0	
Guest	ΔH (kJ/mol)	ΔS (J/mol K)	ΔH (kJ/mol)	ΔS (J/mol K)	ΔH (kJ/mol)	ΔS (J/mol K)
L-tyrosine	-26 ± 3	-63 ± 10	-20 ± 3	-41 ± 11	*	*
YIGSR	-33 ± 4	-81 ± 15	-26 ± 2	-65 ± 14	-15 ± 2	-7 ± 3
YGGFL	-25 ± 2	-61 ± 8	-21 ± 3	-46 ± 11	-36 ± 4	-83 ± 23

* The slope is ill-defined and we estimate ΔH and ΔS to be -9 kJ/mol and 0 J/mol K, respectively.

Inspection of free YGGFL and YIGSR binding with β-CD are especially interesting and require additional analysis. First, the enthalpy of binding becomes *more* favorable as we progress from tyrosine (-9 kJ/mol), to YIGSR (-15 kJ/mole) to YGGFL (-36 kJ/mol). Second, the binding entropy becomes *less* favorable as we progress from tyrosine (ca. 0 J/mol K), to YIGSR (-7 J/mol K) to YGGFL (-87 J/mol K). Third, the enthalpy of binding for YGGFL with β-CD is twofold more favorable compared to YIGSR and fourfold greater than tyrosine alone.

When comparing results between α- and β-CD we note there is an order-of-magnitude change between ΔS for YIGSR binding to α- and β-CD and the entropy is significantly less favorable for the α-CD complex. Similarly, the entropy of binding is about 50% less favorable for YGGFL binding with α-CD compared to β-CD. We note also that the enthalpy for YIGSR binding to β-CD is actually less favorable compared to binding with α-CD (-15 vs. -33 kJ/mol); however, the entropy is able to compensate strongly for this lack of favorable enthalpy.

A plot of ΔH vs. ΔS (not shown) provides a means to assess the level of ΔH–ΔS compensation and yields the equilibrium temperature. The linear relationship is given by $\Delta H = -(10.5 \pm 2.5)$ kJ/mol + (264 ± 36) K (ΔS) ($r^2 = 0.9752$). Rekharsky et al. [42] report equilibrium temperatures of 274 ± 60 K, which agrees well with our values of 264 ± 36 K. Thus, because all our experiments were conducted above 264 ± 36 K, changes in ΔH and ΔS are expected to compensate one another.

4. Discussion

The aims of this work are to determine thermodynamic binding parameters for free tyrosine and tyrosine residues in free YGGFL and YIGSR with α– and β-CD and provide a comparison with published work (Table V). The binding of cyclodextrins with phenolic compounds involves nonspecific van der Waals and hydrophobic interactions [40]. Intermolecular hydrogen bonding may also occur involving the phenolic hydroxyl function and/or other portions of the oligopeptides with the secondary and, perhaps, primary hydroxyls on the outer rim of the cyclodextrin. This

Table V. Published thermodynamic values for the binding of tyrosine to α- and β-CD

	K, M^{-1}	ΔH (kJ/mol)	ΔS (J/mol K)
Tyrosine/α-CD[a]	27.4	−2.1	53.8
Tyrosine/β-CD[a]	33	−4	50
Glycine-tyrosine/α-CD[b]	13 ± 0.2	*	*
Glycine-tyrosine/β-CD[b]	101 ± 2	*	*
Tyrosine/β-CD[c]	108	*	*

[a] From Ref. 18; microcalorimetry, 298 K, 0.1 M phosphate, pH 7.4.
[b] From Ref. 19; competitive spectrophotometry, 296 K, phosphate-buffered isotonic saline, pH 7.4.
[c] From Ref. 20; ^{1}H- and ^{13}C-NMR, 304 K, pH 11.
* No data reported.

may help further stabilize the inclusion complex. There are two possible scenarios for cyclodextrin complex formation: (1) inclusion of the guest molecule inside the cavity, and (2) association of the guest with the perimeter of the cyclodextrin ring structure. In the case of the tyrosine residue, NMR studies have demonstrated inclusion of the phenolic side group *within* the cavity [20, 40].

Inoue and coworkers [20, 43] studied the binding of tyrosine and phenylalanine using ^{1}H- and ^{13}C-NMR using chemical shift differences between the complexed and uncomplexed states. The largest chemical shift differences were observed with β-CD and they suggest that the tyrosine aromatic ring is deeply and tightly sequestered within the β-CD cavity. In the case of α-CD, where smaller chemical shift changes were observed, weaker binding compared to β-CD is suggested. This result is consistent with the geometric dimensions of α-CD [9–12], as deep and tight penetration of the aromatic ring is not possible.

Inclusion of the phenolic ring of tyrosine into the α-CD cavity is geometrically limited. The width of the phenyl ring (distance between the δ hydrogen atoms surrounding the hydroxyl function) is 4.3 Å. This dimension would allow the phenyl ring to fit into the wider opening of the α-CD, but not penetrate fully inside as the cavity narrows. However, the β-CD cavity is much wider [9–12] and would allow the entire aromatic ring to fit easily inside. In addition, the entire length of the tyrosine side chain (6.1 Å, distance between the phenolic proton and methylene carbon) can be included within a β-CD cavity. In this situation, additional stabilization for the complex through hydrogen bonding with the other portions of the oligopeptide are clearly possible.

Horsky and Pitha [19] reported a higher binding constant for the glycine-tyrosine/β-CD complex than the glycine-tyrosine/α-CD complex. This agrees well with our results of significantly higher binding constants for oligopeptide/β-CD

complexes compared to the oligopeptide/α-CD complexes. The binding constants recovered for glycine-tyrosine/cyclodextrin systems cannot, unfortunately, be compared to our oligopeptide/cyclodextrin systems directly because of the differences in size, conformation, and functional groups. However, the trend is, again, consistent.

Our fluorescence data show that the YIGSR/β-CD complex is more stable than the YGGFL/β-CD complex, suggesting that changes in the pentapeptide's conformations may be responsible for the strengths of the equilibrium binding constants. Garbay-Jaureguiberry et al. [44] used ^{15}N-NMR to determine the conformation of YGGFL in DMSO-d_6. Analysis of the $^3J_{15N-1H\alpha}$ coupling constants in concert with the Karplus relationship showed that YGGFL formed a 2–5$\beta_{II'}$ turn (cf., Figure 2 in Ref. 44). To the best of our knowledge, similar structural determinations are not available for YIGSR in solution. However, McKelvey et al. [45] have used molecular dynamics with a simulated annealing procedure to assess the conformations available to YIGSR. Based on these computations it appears that, 'the peptide is forming a segment of a right handed α-helix' (cf., Figure 4 in Ref. 45) [45]. Together these results [44, 45] demonstrate that the intrinsic conformation of YIGSR and YGGFL are very different from one another. In particular, the tyrosine residue in native YGGFL is not nearly as accessible as in YIGSR. This explains in part the poorer binding of β-CD to YGGFL.

Inoue and coworkers [20] reported that the phenylalanine/β-CD complex is *more* stable and favored over the tyrosine/β-CD complex. YGGFL contains both tyrosine (Y) and phenylalanine (F) residues. Thus, it is likely there will be binding to both residues. However, if multiple cyclodextrin bindings were to occur to YGGFL, it would be difficult for a lone YGGFL molecule to bind *two* β-CD molecules simultaneously, simply because the outer diameter of β-CD is 15.3 Å and the two aromatic groups (Y and F) are only 8–10 Å apart. Furthermore, for two β-CD molecules to complex simultaneously with a single YGGFL oligopeptide, a great deal of additional system order would be induced. The results in Table IV suggests exactly this (compare entropy values of -7 J/mol K for YIGSR binding to β-CD with -83 J/mol K for YGGFL binding to β-CD).

5. Conclusions

We have reported on the thermodynamic binding parameters of free tyrosine and tyrosine residues in two free oligopeptides with α- and β-CD. The tyrosine binding to α-CD is independent of the size, amino acid sequence, and pH (7.0 vs. 10.0). This is attributed to geometric limitations of the α-CD cavity. In contrast, size and amino acid sequence/structure have significant affects on the tyrosine binding to β-CD. This was demonstrated clearly when comparing YIGSR and YGGFL. The pentapeptide, YGGFL contains a phenylalanine residue which may compete for β-CD binding, affecting tyrosine residue/β-CD binding. YGGFL's native confor-

mation is such that the tyrosine residue is less accessible compared to the residue in YIGSR.

We have demonstrated that a nonspecific receptor, β-CD, has very different binding affinities for the tyrosine residue in oligopeptides and that this is a function of the oligopeptides structure and residue accessibility. In addition, β-CD has an increased affinity for the tyrosine residue in the two pentapeptides studied here when compared to free tyrosine. This result arises because the unincluded residues within the individual pentapeptides are able to interact favorably with the exterior of the cyclodextrin cavity and provide secondary stabilization affects (i.e., hydrogen bonding). This general trend might increase with the number of amino acid residues until one reaches the point where the accessibility of the tyrosine aromatic ring itself becomes occluded by the other amino acid residues.

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