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Effects of Sequence Context on the Binding of Tryptophan-Containing Peptides by the Cucurbit[8]uril-Methyl Viologen Complex

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ABSTRACT

This paper describes a novel assay for measuring the relative extent of peptide binding in a large parallel format, and the use of this assay to explore the effects of sequence context on the binding of tryptophan (Trp)-containing peptides by the synthetic receptor comprising the noncovalent complex between cucurbit[8]uril and methyl viologen (i.e., Q8•MV). The extent of quenching of Trp fluorescence upon binding to Q8•MV was used to measure the relative extent of binding and thus the relative affinities of 104 Trp-containing peptides, in parallel, using a fluorescence plate reader. This study resulted in the remarkable observation that the identity of the amino acid residues at positions adjacent to the Trp binding site has little if any influence on the binding affinity. This finding suggests that Q8•MV should be effective for the recognition of Trp residues within a broad range of peptide sequences.

Keywords: cucurbit[8]uril, viologen, tryptophan, peptide, assay

1. Introduction

Synthetic receptors that can bind to peptides with well-defined affinities and specificities would have enormous value for biomedical science and technology. The recognition properties of *natural* protein receptors may depend on specificity for a certain type of amino acid residue (e.g., N-recognins, kinases, endopeptidases) (*1-3*) or a certain type of peptide sequence or small protein fragment (e.g., antibodies, cell-surface receptors). In any case, it is important to understand how the sequence environment (i.e., neighboring residues) influences binding. Several *synthetic* receptors have been shown to bind peptides and proteins (*4-17*), but in the majority of cases, these receptors are known to bind to a single type of residue, and the effects of sequence context are understood to a limited extent. This paper describes the effects of sequence context on the interactions of tryptophan-containing peptides with the synthetic receptor, cucurbit[8]uril (Q8), and a method that enables this determination in a rapid and parallel fashion.

Q8 is a tire-shaped, macrocyclic oligomer of bis(methylene)-bridged glycoluril, whose hydrophobic inner space and polar rims drive the inclusion of cationic, organic, small molecules (*18,19*). Compared to other cucurbit[n]urils, Q8 has been studied extensively for its ability to bind two guests simultaneously in aqueous solution (*20-29*). Much of our work with Q8 has focused on the molecular recognition of peptides (*30-36*). We have shown that, when bound to methyl viologen (MV) or tetramethyl benzobis(imidazolium) as the first guest, Q8 binds to peptides containing tryptophan (Trp) with preference for Trp at the N-terminal position (Trp-Gly-Gly; Gly = glycine) versus nonterminal (Gly-Trp-Gly) or C-terminal (Gly-Gly-Trp) positions (Figure 1) (*32*, *36*). Others have used this approach for peptide separation and protein assays (*37-40*). We are interested in the efficacy of peptide recognition across a broad array of sequence contexts. Specifically, we want to know how residues adjacent to the Trp

binding site may influence the binding of Q8•MV. For example, can a basic residue such as lysine or arginine positioned to the N-terminal side of Trp approximate the N-terminal ammonium group that is known to stabilize the binding of cucurbit[n]urils to N-terminal aromatic peptides? Do bulky residues interfere sterically with binding? Small peptides are straightforward to synthesize and characterize in small numbers. Ideally, however, we would like to measure and compare the effects of varying the identity of the residues at positions neighboring the Trp binding site to all possible amino acids, and thus we need to synthesize and screen a library of peptides.

An interesting characteristic of the Q8•MV•Trp system is its optical sensing capabilities. We and others have observed that Trp binding is accompanied by the growth of a new charge-transfer absorbance and the quenching of indole fluorescence (*36*, *41*). Here we present an assay that uses these supramolecular and optical properties to compare in parallel the extent of binding of Q8•MV to a library of 104 Trp-containing peptides by comparing the relative extents of fluorescence quenching. Remarkably, we observe no significant effect of sequence context on Trp binding.

2. Results and Discussion

2.1. Design

The peptide library (Figure 2) was designed such that each peptide contained a tryptophan binding site at either an N-terminal or non-terminal position in order to account for the expected difference in affinity due to the location of the Trp residue (*42*). The C-terminal position was not investigated because it is the lowest affinity site. The N-terminal Trp-containing peptides were tripeptides of sequence Trp-Var_1-Var_2, and the non-terminal Trp-containing peptides were pentapeptides of sequence Var_1-Var_2-Trp-Var_3-Var_4. The variable (Var) positions were each varied among 18 genetically encoded amino acids (*43*) while holding

the other position(s) constant with Ala residues. Ala was chosen as the spacer because its beta-methyl group is the largest sidechain fragment that represents the structures of the other amino acids (all except Gly). This design resulted in a library of 104 peptides (*44*). The peptide library was synthesized by parallel solid-phase synthesis on Rink amide resin (see Supporting Information).

2.2. Parallel Peptide-Binding Assay

The peptide-binding assay is based on the built-in optical sensing capability of the Q8•MV•Trp system, in which the binding of Trp to Q8•MV results in the quenching of indole fluorescence with a linear correlation between the observed extent of fluorescence quenching and the fraction of indole-containing compound bound to Q8•MV (calculated from the known binding affinity (*36*)). This property therefore allows us to estimate the fraction of Trp-containing peptides bound to Q8•MV (and thus the binding affinity) by comparing the fluorescence intensities of each peptide in the presence and absence of Q8•MV (Figure 3). Here we use this approach in the design of an assay to rapidly screen for the relative binding affinity of the library of 104 peptides to the Q8•MV complex. Therefore, this approach is amenable to parallel screening of peptide binding using a fluorescence plate reader.

Figure 4 shows the relative fluorescence quenching (*45*) of the 35 tripeptides (Figure 4a) at 13 μM and the 69 pentapeptides at 30 μM (Figure 4b) in the presence of 50 μM Q8•MV complex (Figure 4b). In the tripeptide series, the extent of fluorescence quenching was in the range 40%-49%, with an average of 45% and a standard deviation of 2%. In the pentapeptide series, the extent of fluorescence quenching was in the range 26%-47%, with an average of 35% and a standard deviation of 3%. In each series, the quenching values are remarkably consistent overall, which indicates that the sequence context of the Trp-binding site has little influence on the binding affinity. This is particularly true for the

tripeptide series, with Trp at the N-terminal position. In the pentapeptide series, the range is larger due to the outliers Lys-Ala-Trp-Ala-Ala (**1**) with 28 % quenching, Ala-Lys-Trp-Ala-Ala (**2**) with 47 % quenching, and Leu-Ala-Trp-Ala-Ala with 26 % quenching. In order to quantify the range of binding affinities represented by the outlier peptides, we measured the equilibrium association constant (K_a) values for the sequence isomers **1** and **2** in complex with Q8•MV.

2.3. Equilibrium Binding Titrations

Figure 5 shows fluorescence titrations of Q8•MV against a constant concentration of peptide for peptides **1** and **2**. The K_a values were 6.2 (±0.3) x 10^3 M^{-1} for peptide **1** and 1.7 (±0.1) x 10^4 M^{-1} for peptide **2**. The small relative binding affinity between these two peptides (2.7-fold, 0.6 kcal mol^{-1}) is measurable but insignificant, and therefore these results further underscore the minimal effect of sequence context on binding to Trp-containing peptides. Using these values in addition to the affinity of Q8 for MV (9 x 10^5 M^{-1}) (*36*) we calculate the difference in the fraction of peptides **1** and **2** bound to Q8•MV to be 17%, which is very similar to the observed difference in fluorescence quenching of 19%, as expected. These results also demonstrate that the assay is sufficiently sensitive to report small differences in binding energies as relatively large differences in the extent of fluorescence quenching.

2.4. Conclusions

This study explores the effects of sequence context on the binding of Q8•MV to Trp-containing peptides. We observe that the identity of the amino acid residues at positions adjacent to the Trp-binding site, for both N-terminal and non-terminal Trp, have little if any influence on the strength of interaction. This result is remarkable given the range of functional groups present in proximity to the Q8 portal, including sidechains with ammonium, carboxylate, and hydrophobic groups, all of which would be expected to influence the binding

affinity via electrostatic, hydrophobic and/or steric interactions. Therefore, Q8•MV should be able to bind Trp residues in a broad range of peptide sequence contexts with predictable binding affinities. This property may prove useful for targeting Trp residues commonly found at hotspots in protein-protein interactions (*15*) and for quantifying surface-exposed tryptophan residues. This study also demonstrates a powerful approach to the parallel screening of peptide interactions using a synthetic receptor using the convenient measurement of fluorescence intensities to estimate relative binding affinities. This approach would be compatible with a strategy for altering the binding properties of the receptor, Q8•MV, by tailoring the structure of the viologen cofactor. We are currently exploring this direction and will report those results in due course.

3. Materials and Methods

3.1. Instrumentation

UV-visible spectra were acquired at 25 °C using an Agilent 8453 spectrophotometer. Electrospray ionization mass spectrometry (ESI-MS) was performed on a Thermo LCQ DECA XP mass spectrometer with an electrospray ion source in the positive ion mode. Fluorescence quenching and titration experiments were carried out using a Tecan Infinite M200 Pro plate reader, with 280 nm excitation and 350 nm emission wavelengths, 5 nm excitation bandwidth, 20 nm emission bandwidth, and 20 μs integration time. The fluorescence spectra of Trp-Ala-Ala and Q8•MV•Trp-Ala-Ala were collected on a PTI QM-4 spectrophotometer with 280 nm excitation wavelength, 3 nm excitation slit width, and 5 nm emission slit width.

3.2. Materials

The following compounds were of analytical purity grade and used without purification: (L)-Fmoc-Ala-OH, (L)-Fmoc-Arg-(Pbf)-OH, (L)-Fmoc-Asn(Trt)-OH, (L)-Fmoc-Asp(OtBu)-OH, (L)-Fmoc-Cys(Trt)-OH, (L)-Fmoc-Glu(OtBu)-OH, (L)-Fmoc-Gln(Trt)-OH, (L)-Fmoc-Gly-OH, (L)-Fmoc-His(Trt)-OH, (L)-Fmoc-Ile-OH, (L)-Fmoc-Leu-OH, (L)-Fmoc-Lys(Boc)-OH, (L)-Fmoc-Met-OH, (L)-Fmoc-Phe-OH, (L)-Fmoc-Pro-OH, (L)-Fmoc-Ser(tBu)-OH, (L)-Fmoc-Thr(tBu)-OH, (L)-Fmoc-Trp(Boc)-OH, (L)-Fmoc-Tyr(tBu)-OH, (L)-Fmoc-Val-OH, and O-(benzotriazol-1-yl)-N,N,N′,N′-tetramethyluronium hexafluorophosphate (HBTU) (Peptides International); biotech-grade dimethyl formamide (DMF), diisopropylethylamine (DIEA), triisopropylsilane (TIS), trifluoroacetic acid (TFA), piperidine, anhydrous dichloromethane (DCM), and HPLC-grade acetonitrile (Sigma-Aldrich); and monobasic and dibasic sodium phosphate (VWR). Cucurbit[8]uril (Q8) was synthesized according to a published procedure (*46*). Water was obtained from a Barnstead Nanopure Infinity water system (18 MΩ cm).

A stock solution of 1.0 M sodium phosphate buffer was adjusted to pH 7.0 and sterile filtered. 10 mM sodium phosphate buffer was made as needed by diluting the 1.0 M stock and adjusting to pH 7.0. The concentration of methyl viologen was determined by UV spectroscopy (ε_{257} = 20,400 $M^{-1}cm^{-1}$). The concentration of Q8 was standardized by calorimetric titration with methyl viologen.

3.3. Peptide Synthesis

Parallel fmoc solid-phase synthesis was carried out using SynPhase Rink amide Lantern resins from Mimotopes on 8 µmol scale. The resins were mounted to pins in an 8 x 12 array (i.e., the "rack of resins") to match the spacing of a standard 96-well plate. Fmoc deprotection was accomplished by adding 500 µL of 20% piperidine (v/v) in dimethylformamide (DMF) into each well of a 96 well deep-well block (Hamilton Research), and then seating the rack of resins into the wells of the block and allowing the reaction to shake at 120 RPM for 1 hour 25 °C in an orbital shaker (e.g., a shaking bio-incubator). The rack of resins was then rinsed thoroughly according to the following procedure: 1) two baths of DMF and dichloromethane (DCM) were set up; 2) the rack of resins was rinsed thoroughly and sequentially by dipping first into the two DMF baths, and then into the two DCM baths, with gentle flicking of the rack between each rinse to remove excess solution; and 3) the rack of resins was removed and allowed to air dry for approximately 20 minutes.

To prepare benzotriazoyl-activated amino acid solutions for coupling, we used 12 equivalents (96 µmol) of fmoc amino acid, 12 equivalents (96 µmol) of di-isopropyl ethylamine (DIEA), and 10 equivalents (80 µmol) of HBTU (based on a limiting quantity of 8 µmol resin) dissolved in 0.5 mL of DMF. Amino acid solutions were scaled to the number of couplings needed per amino acid in the entire library so that only one stock solution of each amino acid needed to be

prepared. The coupling reaction was accomplished by adding 0.5 mL of the activated amino acid solution into a well of a clean 96-well deep-well block and then seating the resin rack into the block. The reaction shook (120 RPM) for 3 hours at 25 °C, and the resins were then rinsed as described above.

Cycles of deprotection and coupling continued in this manner until the desired sequence was obtained. After the final fmoc deprotection, the resins were rinsed as described above. Cleavage of the peptides from the resins was carried out by adding 600 µL of cleavage solution (95% TFA, 2.5% H_20, 2.5% TIS), into each well of a clean 96-well deep-well block, seating the resin rack into it, and letting the reactions shake at 120 RPM for 1 hour at 25 °C. The resin rack was removed, and the cleavage mixture was evaporated overnight with steady airflow over the plate. After the TFA was removed completely, 800 µL of nanopure water was added to each well, and the block was heated at 60 °C for 30 min to dissolve the peptides. The peptide solutions were transferred to 1.5 mL Eppendorf tubes and lyophilized to dryness. The dry peptides were resuspended in 1.0 mL of 10 mM phosphate buffer, heated for 15 minutes at 60 °C, and sonicated if necessary to solubilize the peptides. A random subset of six peptides was tested for purity, quantity, and identity (See Supporting Information). The average purity, as determined by analytical HPLC, was 78%. The average concentration, as determined by UV-spectroscopy (ε_{280} = 5500 M^{-1} cm^{-1}) was 2.1 mM for the tripeptides and 4.8 mM for the pentapeptides, and thus the peptide recoveries from synthesis were 26% for the tripeptides and 60% for the pentapeptides. The identities of the peptides were confirmed by ESI-MS. The peptide solutions were stored at 4 °C.

3.4. Fluorescence Quenching Experiments

Q8•MV solutions were prepared by dissolving MV in 10 mM sodium phosphate buffer, pH 7.0, and determining the concentration of the solution by UV

spectroscopy (ε_{257} = 20,400 $M^{-1}cm^{-1}$). This solution was adjusted to 100 μM in the same buffer and added to an equimolar quantity of dry Q8. The Q8•MV mixture was solubilized by mixing and brief ultrasonication followed by heating at 60 °C for 15-20 minutes. The resulting colorless solution was cooled to room temperature and sterile-filtered (0.4 μm, Teflon).

Fluorescence experiments were carried out in Corning 96-well, black, flat-bottomed plates. Peptides sample solutions were prepared by mixing 50 μL of peptide stock solution with 50 μL of 10 mM phosphate buffer. Peptide + Q8•MV solutions were prepared by mixing 50 μL of peptide stock solution with 50 μL of 100 μM Q8•MV solution. The peptide stock solutions were 26 μM for tripeptides and 60 μM for pentapeptides. Therefore, the final concentrations were 13 μM tripeptide or 30 μM pentapeptide, and 50 μM Q8•MV for samples that contain Q8•MV. The solutions were mixed by pipetting before reading the fluorescence intensity on a fluorescence plate reader. The extent of fluorescence quenching (%Quenched) was determined as the fraction of fluorescent emission intensity (F) lost upon treatment with Q8•MV:

$$\%Quenched = \frac{(F_{peptide} - F_{peptide+Q8\bullet MV})}{F_{peptide}} \qquad \text{Eq. 1}$$

3.5. Fluorescence Titrations

Fluorescence titrations were carried out on peptides **1** and **2**. The peptides were quantified UV spectroscopy (ε_{280} = 5500 M^{-1} cm^{-1}). In the final samples for measurement, the concentration of peptide was held constant at 0.121 mM for peptide **1** and 0.206 mM for peptide **2**, while the concentration of Q8•MV was varied over the range 0-1.4 mM. Fluorescence intensity values were obtained using the plate reader and plotted versus the total peptide concentration. Each plot was fit to a simple binary equilibrium model using a nonlinear regression to obtain equilibrium association constant values.

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(42) Based on prior work (*36*), equilibrium association constant (K_a values are ~10^5 for N-terminal and ~10^4 for non-terminal peptides.
(43) Tryptophan and cysteine (Cys) were not included in the variation because the presence of a second Trp binding site and fluorescent marker would complicate the analysis, and because Cys-containing peptides spontaneously dimerize.
(44) Leaving out the replicate Trp-Ala-Ala and Ala-Ala-Trp-Ala-Ala peptides (i.e., Var_1=Var_2=Ala), there are 35 tripeptides and 69 pentapeptides.

(45) The extent of fluorescence (F) quenched (%Quenched) was calculated from Equation 1.
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Figure Captions

Figure 1. (top) Equilibria involved in the formation of the Q8•MV•Trp complex. (bottom) Chemical formulas of the constituents.

Figure 2. Library of (left) tripeptides and (right) pentapeptides used in this study. The variable (Var) positions were varied to 18 amino acids (the canonical 20 minus Trp and Cys).

Figure 3. Fluorescence spectral overlay of Trp-Ala-Ala in the absence and presence of Q8•MV. All species were at 50 μM in a buffer of 10 μM sodium phosphate, pH 7.0.

Figure 4. Extent of fluorescence quenching of samples containing (a) 13 μM tripeptide or (b) 30 μM pentapeptide in the presence of 50 μM Q8•MV at 25 °C in 10 mM sodium phosphate, pH 7.0. The identity of the variable position is indicated along the Y-axis, and the location of the variable positions within the peptides are indicated by the legends. Average values of at least three experiments are plotted. Error bars indicate the standard deviation.

Figure 5. Titration of Q8•MV against a constant concentration of peptides **1** (0.121 mM) and **2** (0.206 mM). Relative fluorescence emission intensity values are plotted. The line indicates the best fit to binary equilibrium binding model. Equilibrium association constant values, derived from the fits, are shown.