Surface Recognition and Helix Stabilization of a Tetraaspartate Peptide by Shape and Electrostatic Complementarity of an Artificial Receptor

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Abstract: The recognition of tetraaspartate peptide (**2**) by tetra-guanidinium (**1**) in (a) water and (b) aqueous methanol (10% H_2O/90% CH_3OH) has been investigated. The conformations of the free peptide and its complex with **1** have been characterized in both solvent systems by CD and 2D NMR spectroscopies. Increased α-helicity as a result of solvent composition and receptor binding are discussed. Control experiments show that the recognition stems from complementary electrostatic interactions, hydrogen bonding, and matching of surface topologies.

Introduction

Protein−protein interactions are governed by their macromolecular architectures.[1] A matching of surface topologies between the interacting species arises from shape (mainly hydrophobic surfaces) and functional group complementarities. Hydrogen bonds and electrostatic forces play a crucial role in these interactions.[2] The carboxylate anion−guanidinium cation interaction is especially important because it forces a complementary ion-pair interaction with the formation of two hydrogen bonds. In nature, charged groups such as carboxylates and guanidiniums are present on the surface of proteins not only to help solubilize these large molecules in an aqueous environment, but also to play a significant role in orchestrating the selectivity of molecular interactions.

To better understand protein−protein interactions as well as to design receptors able to perform efficient and selective protein recognition, several groups have devoted efforts toward defining and evaluating model systems for the recognition of protein surfaces.[3] Our efforts originated in the wealth of information already obtained through the design of guanidinium-based carboxylate/anion receptor molecules.[4] Prior studies had shown that tetraguanidinium receptor **1** (Figure 1) presents,[5] due to its tetracationic character, a cooperative binding face to polyanionic ligands with a consequent increase in binding affinity and selectivity. Also, when all stereogenic centers in the molecule have the same configuration (*all-R* in compounds **1** and **3**) the compound tends to adopt a helical conformation. Finally, the compound is relatively rigid since when chloride is replaced as counterion by divalent sulfate,[5] helical, bridged dimers are formed. Consequently, these guanidinium-based receptors are good candidates for the specific recognition of an $i + 3n$ ($n = 0, 1, 2, 3$) spaced tetra-carboxylate sequence presented on the helical surface of a peptide (Scheme 1).

We have previously reported that a synthetic tetraguanidinium derivative was able to interact with a tetraaspartate peptide and to promote an increase of helicity in aqueous methanol (10% H_2O/90% CH_3OH) as shown by CD and preliminary NMR experiments.[6] Here we report more details on the spectroscopic characterization of the complex in (a) water and (b) aqueous methanol (10% H_2O/90% CH_3OH) and provide data indicating that even in water there is some peptide−receptor interaction and a resultant increase in helicity. The target peptide **2** was designed to contain four anionic Asp residues at positions $i + 3n$ ($n = 0, 1, 2, 3$) to match the four guanidinium groups of receptor **3**. Molecular modeling studies had previously shown that the charged aspartates of **2** in an α-helix could be recognized by tetraguanidinium **3** in a "super helical" arrangement.[6] This mode of recognition also induces α-helicity in the peptide sequence. Helices derived from L-amino acids are right-handed and are characterized by a periodicity of 3.6 residues per turn with a helical pitch of 5.4 Å.[7] Residues at $i + 3n$ are positioned in a left-handed "super-helical" arrangement with a pitch of ca. 27 Å.

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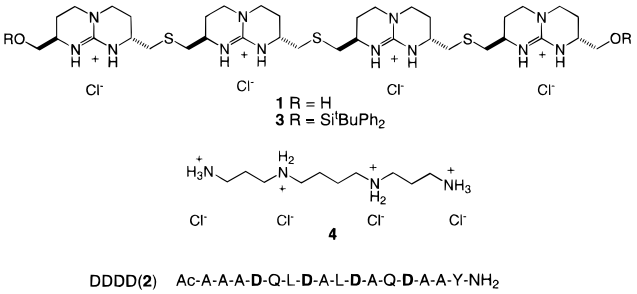
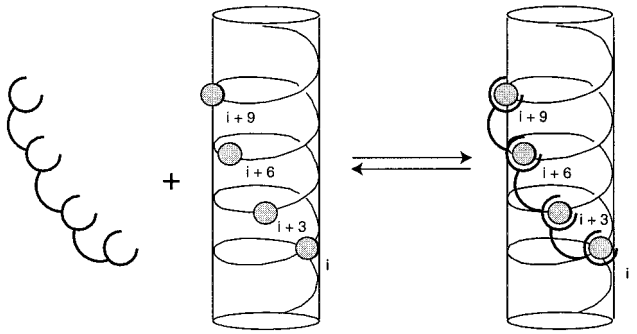


DDDD(**2**) Ac-A-A-A-**D**-Q-L-**D**-A-L-**D**-A-Q-**D**-A-A-Y-NH_2

Figure 1. **1** and **3**: Tetraguanidinium molecules used in binding and structural studies. **2**: Tetraaspartate peptide DDDD. **4**: Spermine.

Scheme 1. Schematic Representation of Tetraguanidinium Binding to an α-Helical Tetraaspartate Peptide with an $i + 3n$ ($n = 0, 1, 2, 3$) Spacing of Aspartate Residues



Herein we report conformational studies using CD and high-field 2D-NMR at variable temperatures to analyze the nature of the binding interaction between peptide **2** and tetraguanidinium receptor **1** as well as the resultant helicity in the peptide due to complexation.

Materials and Methods

Synthesis/Physical Data. Peptide **2** was synthesized using standard solid-phase peptide synthesis techniques and purified by reversed-phase HPLC.[8] Physical data for peptide **2**: Ac-A-A-A-D-Q-L-D-A-L-D-A-Q-D-A-A-Y-$CONH_2$. EMS: Calcd: 1661.7. Found: 1661.2. Amino acid analysis (AAA): $Asx_{4.3}Gln_{2.1}Ala_{6.8}Leu_{2.0}Tyr_{1.0}$.

Peptide **2** was analyzed as the tetramethylammonium (TMA) salt. It was generated from the corresponding acid by dissolving in water and adjusting the pH to 10.5−11.0 with concentrated ammonium hydroxide solution and subsequent lyophilization. The resulting sample was then dissolved in H_2O and 1−2 mL of tetramethylammonium cation-exchange resin (Amberlite IRA-120) were added. The solution was stirred for 8−12 h and the exchange resin filtered and re-lyophilized to give the corresponding tetramethylammonium salt.

CD Binding Titrations/K_a Determination/Percent Helicity Calculations. CD samples were studied in the following solvents: H_2O, 10% H_2O/CH_3OH, or 10 mM buffered 10% H_2O/CH_3OH. Buffers used were 2-(*N*-morpholino)ethanesulfonic acid (MES) at pH 6, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid (HEPES) at pH 7 and 8, and 2-(*N*-cyclohexylamino)ethanesulfonic acid (CHES) at pH 9 and 10. The buffered samples were made by dissolving the peptide in CH_3OH, and diluting to a solvent composition of 10% H_2O/CH_3OH with 100 mM buffer. This made the concentration of buffer equal to 10 mM in the H_2O/CH_3OH mixture. The pH readings reported are the uncorrected meter readings of the buffered solutions in 10% H_2O/CH_3OH.

Peptide concentration was determined based on the UV absorbance of tyrosine in 6 M guanidine hydrochloride, 100 mM phosphate at pH 7.0, $\epsilon_{276} = 1450\ M^{-1}\ cm^{-1}$.[9] Titrations were conducted at a constant concentration of peptide (50 ± 10 μM) with addition of receptor from 0 to 250 μM with CD monitoring at 222 nm. Binding data were analyzed by following the change in magnitude of the CD signal at 222 nm upon addition of increasing amounts of receptor. The CD signals of the receptor over the concentration range were subtracted from the titration CD signal to give the signal inherent to the peptide in solution. To do this, samples ranging in concentration from 0 to 250 μM of the guanidinium receptor in 10% H_2O/CH_3OH were collected independently and subsequently subtracted.

Ellipticity is reported as mean residue ellipticity. Percentage (or fraction, *f*) of helicity was determined from the following equation:

$$f = 100[(\Theta_{obs} - \Theta_0)/(\Theta_{100} - \Theta_0)]$$

Θ_{obs}, Θ_0, and Θ_{100} represent the measured ellipticity, the zero ellipticity (random coil), and 100% helicity (as calculated), respectively, and *f* is the percent (or fraction, *f*) of helix calculation of 100% helicity following the equation of Charkrabartty et al.[10] Zero percent helicity was determined experimentally by thermal denaturation of peptide **2** in pure water. The measurement gave −2060 ($deg \cdot cm^2 \cdot dmol^{-1}$) and was used as Θ_0 in the percent helix calculations.

Curve fitting was done using a 1:1 binding model by nonlinear curve-fitting analysis on Kaleidograph software for the Apple Macintosh. Fitting to the CD signal at 222 nm as a function of receptor concentration in M gave the K_a values in M^{-1}. A 1:1 binding stoichiometry was confirmed by a Job plot. A titration of continuous variation was conducted where the concentration of both peptide **2** and receptor **3** was varied between 0 and 100 μM such that the number of moles in solution stayed constant. A plot of the mole fraction of peptide **2** in the solution vs ($-\Delta_{MRE}$ × mole fraction of **2**)[11] gives a plot that indicates the stoichiometry of the binding event. Δ_{MRE} is defined as the difference between the observed signal of the species in the titration and its inherent signal.

NMR Spectroscopy. Samples were prepared using a concentration of 2 mM peptide in 600 μL of either (A) 85% H_2O/15% D_2O or (B) 90% d_3-methanol/10% H_2O. Dioxane was used as internal standard. ^1H NMR spectra were recorded on a Varian VXR-500 unit equipped with a shielded triple resonance gradient unit. 2D-homonuclear NMR spectra were recorded in phase-sensitive mode using the States method for quadrature detection in the t1 dimension. DQF-COSY[12] spectra were recorded with 1.5 s of presaturation. Clean-TOCSY[13] experiments were recorded with a MLEV-17 spin-lock sequence[14] at a mixing time of 80 ms, and NOESY[15,16] were recorded with mixing times of 100, 200, and 400 ms to evaluate spin-diffusion effects. ROESY[17] spectra were acquired at low mixing times (150 ms) to differentiate between ROE connectivities and chemical exchange peaks. Solvent suppression was achieved for TOCSY and NOESY/ROESY using a WATERGATE sequence.[18] The acquisition time for two-dimensional spectra was in general set to 0.2 s, followed by a recovery delay of 1.6 s. A spectral width of 5000 Hz was used in both dimensions containing 2048 to 1024 data points. Usually 32 or 64 scans for 256 increments were recorded to achieve sufficient sensitivity and resolution. Spectra were processed with the Varian built-in software package VNMR on a SUN-

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Table 1. Binding Affinities and Percentage Helicities for Peptide−Receptor Interactions

solvent	receptor	K_a, M^{-1}	H_i[a]	H_f[b]
10% H_2O/90%CH_3OH	**1**	$3.4 \pm 1.2 \times 10^5$	21.1	45.3
10 mM MES pH 6 in 10% H_2O/ 90%CH_3OH	**1**	$5.8 \pm 5.4 \times 10^4$	33.8	40.5
10 mM CHES pH 9 in 10% H_2O/ 90%CH_3OH	**1**	$1.9 \pm 0.8 \times 10^5$	13.1	46.9
10 mM CHES pH 10 in 10% H_2O/ 90%CH_3OH	**1**	$1.8 \pm 0.8 \times 10^5$	11.5	48.1
10% H_2O/90%CH_3OH	**4**	$2.4 \pm 1.9 \times 10^5$	32.7	26.7

[a] Initial percentage helicity.[10] [b] Percentage helicity at 2 equiv of receptor.

SPARC work station. Zero-filling or linear prediction of the t1 dimension results in a matrix of 2 × 4K, which was baseline corrected in both F1 and F2 dimensions and multiplied by either a shifted Gaussian or sine-bell function prior to the Fourier transform. Approximate $^3J_{\alpha N}$ coupling constants were measured from 1D spectra or after analyzing the corresponding 1D slices from resolution enhanced DQF-COSY spectra with a 4K × 1K real data-point matrix. Temperature coefficients of the amide protons were extracted from high-resolution 1D or fast-TOCSY (256 increments containing 8 transients with 2048 data points each) in 5 deg intervals from 278 to 308 K.

Results

Two solvent systems, water and aqueous methanol (10% H_2O/ 90% CH_3OH), were chosen to study the binding of receptor **1** to peptide **2**, focusing on CD and NMR conformational analysis of the peptide. The aqueous methanol acted as a secondary structure stabilizing solvent in a manner similar to trifluoroethanol or hexafluoro-2-propanol. CD binding titration data are listed in Table 1. NMR assignment was done based upon the standard two-step procedure using a combination of TOCSY and NOESY (ROESY) spectra.[19] To study whether aggregation was taking place in the NMR conditions, 1D ^{1}H NMR spectra of both peptide **2** and the complex formed between peptide **2** and receptor **1** were recorded at three different concentrations (10 μM, 100 μM, and 1 mM).[20] The fact that there were no changes either in chemical shift or line width shows that the species present in the NMR conditions are monomeric. We will present the results of the peptide sequentially in the absence and presence of the tetraguanidinium derivative **1**.

Free Peptide in Water. In water, peptide **2** shows no evidence for the formation of regular secondary structure by either CD or NMR spectroscopies. The CD spectrum (Figure 2) shows a single minimum around 200 nm typically observed for random coil peptides. The NMR spectra over a range of temperatures show small amide chemical shift dispersions (Figure 3A), Hα conformational shifts close to random coil values (Figure 4), the absence of amide−amide or medium (long) range NOEs or ROEs (Figure 3A), and $^3J_{\alpha N}$ coupling constants between 6 and 8 Hz. Only the C-terminal residues Ala15-Tyr16 and Asp4-Gln5 were unequivocally identified without using data from the putative complex. Assignment was done by comparison with the peptide in the presence of the tetraguanidinium derivative **1**.

Free Peptide in Aqueous Methanol. In aqueous methanol, the CD spectrum shows the appearance of two minima at 208 and 222 nm and a maximum at ∼195 nm (Figure 2), characteristic of an α-helical conformation. A series of strong sequential NN(i, i + 1)/αN(i, i + 1) (Figure 3B, 4) NOEs from Ala 3 to Tyr 16, several medium range NN(i, i + 2), αN(i, i + 3) NOEs, and $^3J_{\alpha N}$ coupling constants below 6 Hz are consistent with helical conformations in this region. Furthermore, negative Hα conformational shifts (HαCS) throughout the sequence are an indication of helicity. Calculations based upon CD absorption at 222 nm[10] and HαCS[21] gave by both techniques a total helical content of 21−22%. This helicity in aqueous methanol (hereby termed "inherent helicity") is in agreement with some predisposition for α-helicity of peptide **2**, whose design was based upon the Baldwin polyalanine sequence motif.[22] HαCSs show another peculiarity, namely an i + 3 periodicity from Asp 7 toward the carboxy terminus with minima for the acidic residues Asp 7, 10, and 13 (Figure 4A). Periodicity in conformational shifts is typically observed for curved and/or amphiphilic helices with hydrophobic and hydrophilic faces.[23] Additional evidence for amphiphilic helices is normally derived from the i + 3 periodicity of the amide conformational shifts.[23] As shown in Figure 5B, amide conformational shifts (ACS) do not show the same pattern as Hα conformational shifts but correlate to some extent (anticorrelation coefficient $R = 0.769$) with their corresponding temperature coefficients (ATC). In particular, largely negative ATCs are correlated with low field shifted amide protons. Usually, largely negative amide ATCs are interpreted as evidence for solvent-exposed amide protons and thus, long or weak H bonds.[24] On the other hand, it is widely accepted that low field shifted amide protons are associated with strong, short hydrogen bonds. This surprising lack of consistency can be explained, however, assuming the two-state random coil helix equilibrium and observing the effect of temperature upon the conformational ensemble.[25,26] Only peptides lacking a two-state conformational equilibrium, such as completely flexible peptides

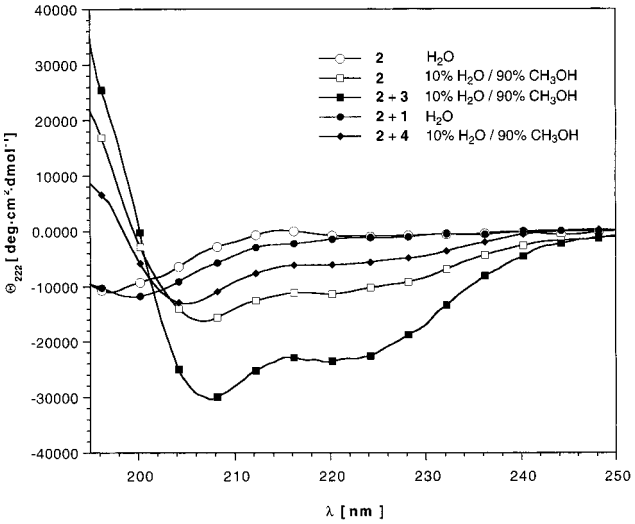

Figure 2. CD spectra of peptide **2**: in water (○), in 10% H_2O/90% CH_3OH (□), in water with 2 equiv of **1** (●), in 10% H_2O/90% CH_3OH with 2 equiv of **3** (■), and in 10% H_2O/90% CH_3OH with 2 equiv of **4** (◆).

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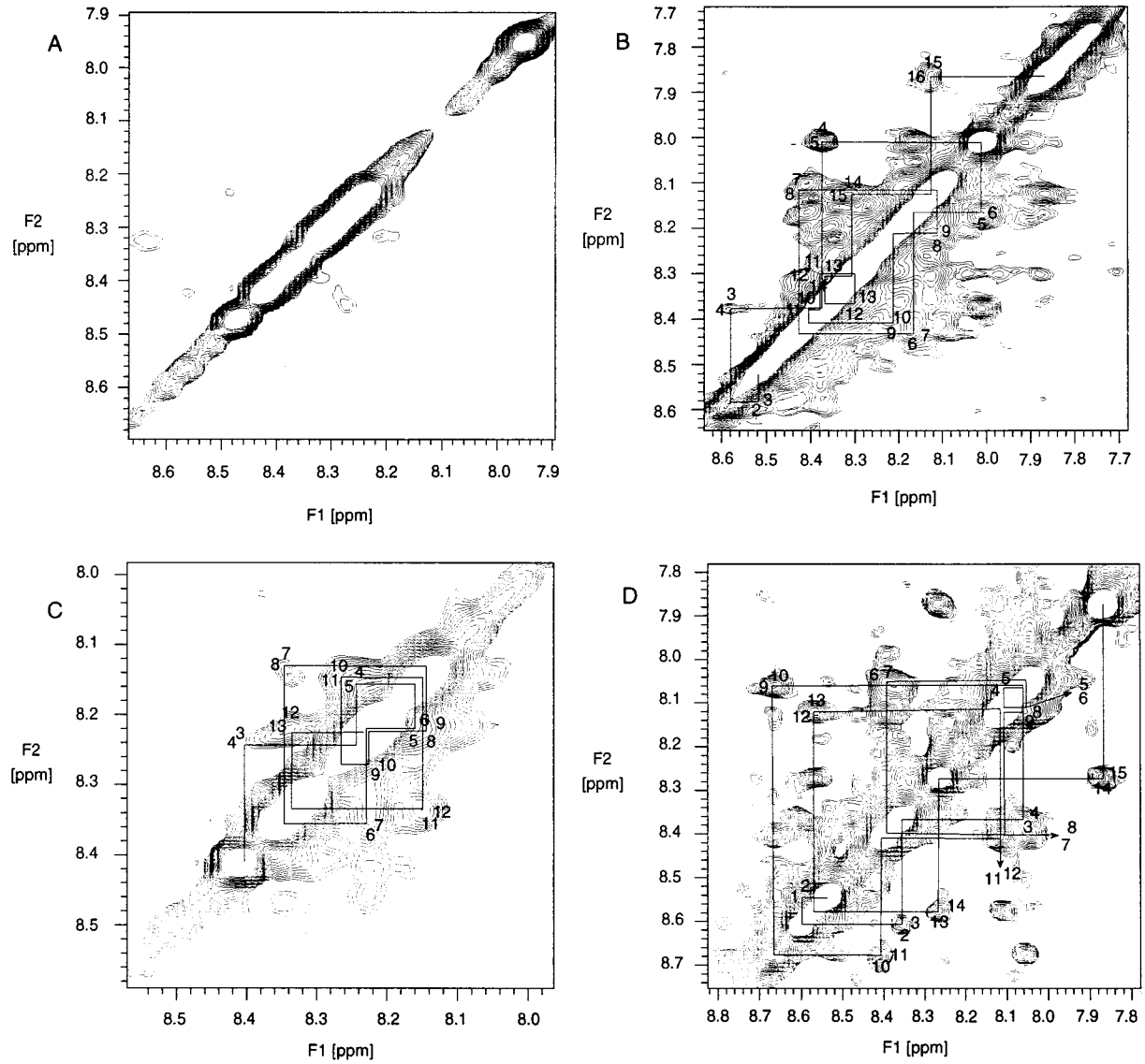

Figure 3. Expansion of the aromatic and amide protons region of the NOESY (300 ms) ^{1}H NMR spectra of **2**: (A) in water, (B) in 10% H_2O/90% CH_3OH, (C) in water in the presence of 1equiv of **1**, and (D) in 10% H_2O/90% CH_3OH in the presence of 1 equiv of **1**. Residue numbers refer to the sequence of **2**.

without any structural preference or peptides involved in stable protein structures, do not show the above-mentioned ACS/ATC correlation. In these cases, ATCs can be used to derive solvent protection factors. Thus, the absence of ACS $i + 3$ fluctuation and the presence of ATC/ACS correlation is in accordance with the presence of a helix in a two-state helix-coil equilibrium, according to the proposed model.

Conformational analysis of peptide **2** in the presence of equimolar amounts of receptor **1** shows several indications of a higher amount of α-helical secondary structure by NMR and CD.

Peptide/Receptor Complex in Water. In water, the extent of NOEs significantly increases after addition of the receptor, as shown in Figure 3C. NN(i, $i + 1$) NOEs or ROEs were not detected in the absence of the receptor, but clearly appeared when it was present. Under these conditions, the small chemical shift dispersion prevents the detection of medium range NOEs strongly overlapping with sequential NOEs. Hα conformational shifts were similar to the noncomplexed peptide with the exception of Leu 6, Asp 7, and Ala 8 (Figure 4A). Helical content calculated by Hα conformational shifts does not give a significant difference in values in the presence or absence of the receptor; similar results were obtained from CD studies (Figure 2). Nevertheless, some $i + 3$ periodicity could be observed for the Hα conformational shift in the presence of the receptor, suggesting helix formation (Figure 4A).

ATCs are not correlated with their corresponding conformational shifts ($R = 0.326$) excluding a coil−helix equilibrium. Therefore, ATCs are used in this case to derive a solvent protection factor and insights into more stable structures. All ATCs, except Asp 7, Asp 10, Asp 13, and Ala 8 are lower in the presence of the receptor than in its absence. This result suggests some degree of amide solvent protection and thus higher secondary structure stability due to the presence of the receptor, even though a specific increase in helicity is uncertain.

(26) Increasing temperature will shift the two-state equilibrium toward the random coil state. Consequently, both HαCS and ACS values will be shifted closer to zero or random coil values. Further, helical conformations are stabilized by intramolecular hydrogen bonds between amide protons and carbonyl oxygens of the backbone. Thus, increasing temperature will increase the lengths of these intramolecular hydrogen bonds. Considering the inverse cubic distance dependence of the amide chemical shift,[25] small changes of temperature will thus strongly affect the amide chemical shifts involved in strong short hydrogen bonds and give rise to large negative ATCs. Solvent accessibility plays only a secondary role under the experimental conditions.

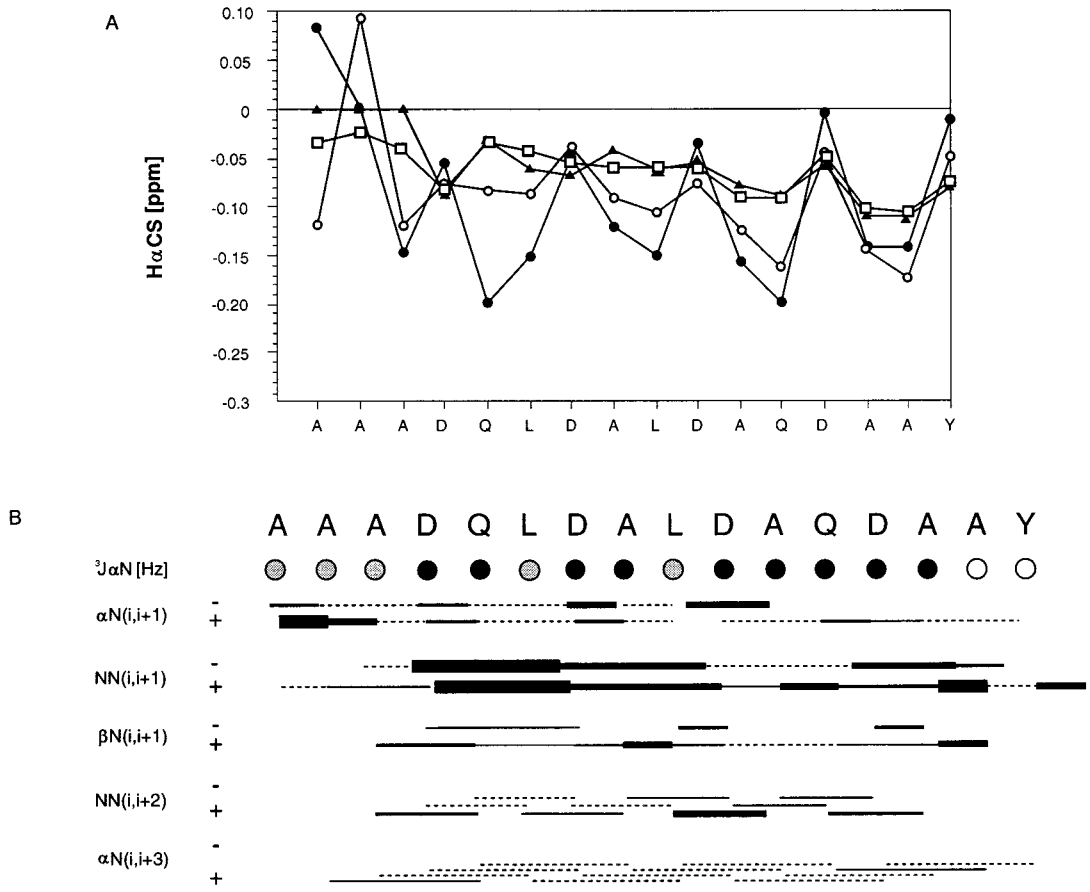

Figure 4. (A) Hα conformational shifts (HαCS) of **2** in water (□), **2** in 10% H_2O/90% CH_3OH (○), **2** in water in the presence of 1 equiv of **1** (▲), and **2** in 10% H_2O/90% CH_3OH in the presence of 1 equiv of **1** (●). (B**)** Summary of the sequential and medium range NOEs observed for **2** in 10% H_2O/90% CH_3OH in the presence (+) and in the absence (−) of 1 equiv of **1**. Relative NOE intensities are represented by the heights of the bars. Overlapping or ambiguous NOEs are indicated by dashed lines. Filled black circles represent $^3J_{\alpha N}$ values of **2** in 10% H_2O/90% CH_3OH below 4 Hz; gray shaded circles represent values between 4 and 6 Hz, and open circles indicate $^3J_{\alpha N}$ above 6 Hz.

Peptide/Receptor Complex in Aqueous Methanol. In aqueous methanol, addition of the receptor provokes an increase (absolute value) of the CD spectrum at 222 nm (Figure 2), an increase in the amide chemical shift dispersion of about 0.15 ppm, and a higher amount of sequential/medium range NOEs (Figure 3D, 4B) in the presence of equimolar amounts of receptor. Intermolecular NOEs between leucine methyl groups and the protons of the sulfur bound methylene spacers give conclusive evidence for the formation of a stable complex between peptide **2** and receptor **1**. Spectral overlap precludes observation of intermolecular NOEs between the side chains of Gln 5 and 12 and the protons of receptor **1**.

Hα conformational shifts from Asp 4 to Tyr 16 are more negative for all non-Asp residues and show a very strong $i + 3$ periodicity with Asp as maxima and Leu or Ala as minima (Figure 4A). Simple calculations of helicity based upon Hα conformational shifts gave 24% including all residues and 41% excluding the Asp residues. In comparison, the free peptide in aqueous methanol shows for the same residues 22% (all residues) or 32% (non-Asp residues), respectively. The increase in helicity upon addition of receptor **1** calculated by NMR was therefore 2% or 9%, depending on whether the Asp residues are included or not. Binding studies by CD show that under the same conditions (equimolar amounts of peptide and receptor **1** at 50 μM) an increase in the absorption at 222 nm occurs which gives an indication of the increase in helicity corresponding to a 24% enhancement.

These discrepancies can have several origins, mainly in the determination of helicity by NMR HαCS analysis. First, HαCSs are defined as the difference of HαCS between experimental and reference values determined for random coil peptides in water[27] and aqueous TFE.[28] The use of solvents other than water or mixtures of water/TFE could lead to a deviation from the correct value. Nevertheless, we observed for the peptide alone an excellent agreement of CD and NMR/HαCS derived helicities in aqueous methanol ruling out an error derived from improperly set reference values. Another important point could be the ionization state of the complexed peptide, which may be different from the peptide in the presence of the TMA counterions. The strong periodicity of the HαCS with positive values in both N and C terminal residues make an alternative explanation most probable. Whereas NOE analysis, ACS/ATC correlation, and coupling constant analysis show the presence of a helix throughout the sequence, HαCS analysis shows negative values only for residues 4−16. Thus, a relatively low helicity is expected from NMR parameters in comparison with CD data when calculating the total helicity over the whole sequence.

ATCs and ACSs were used to derive additional information about the structural state of the peptide in the presence of the receptor. No clear trend was observed when considering the amide temperature coefficients alone but a very high ACS/ATC correlation was found ($R = 0.900$, Figure 5D). As mentioned earlier, this kind of high correlation suggests the formation of

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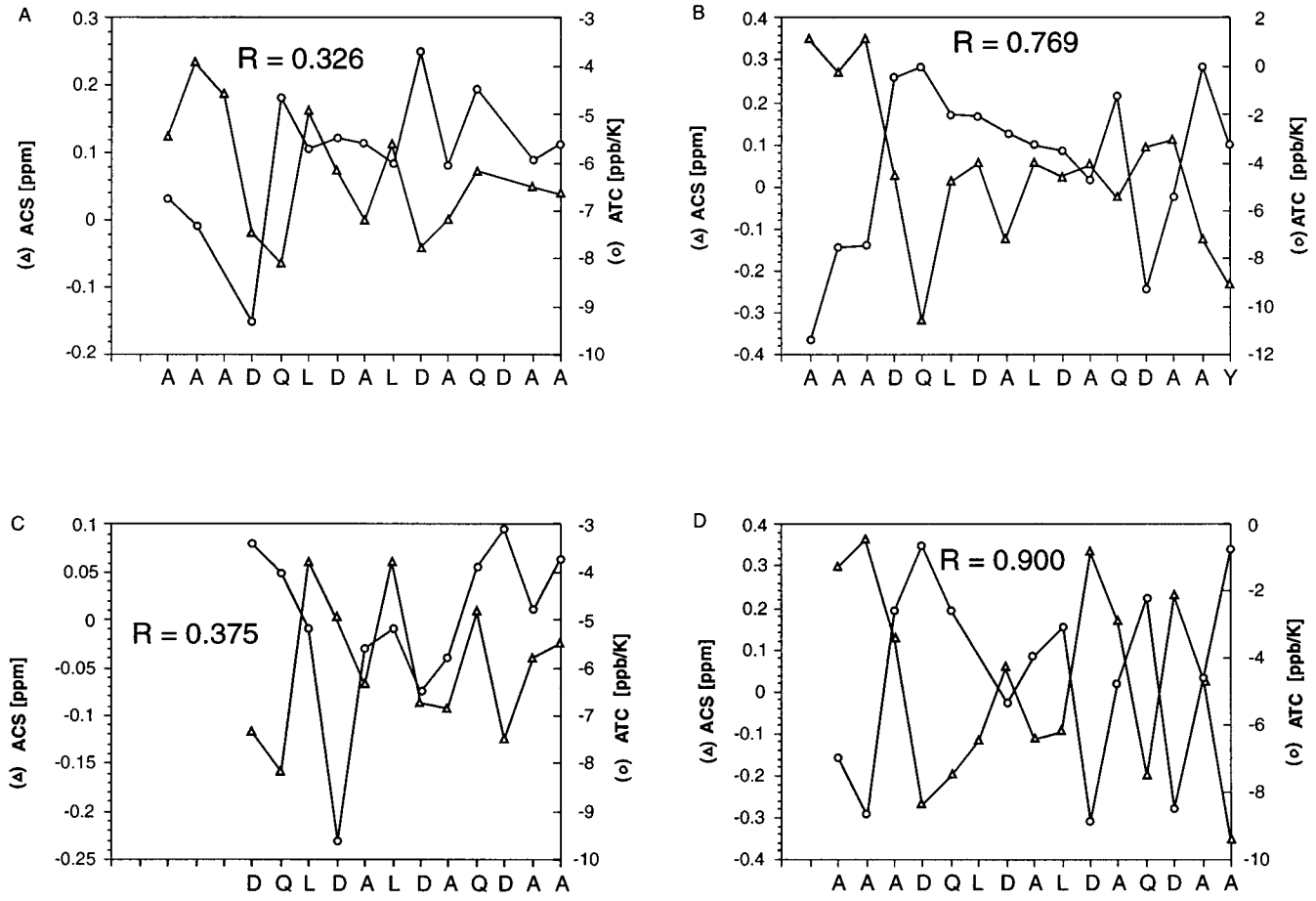

Figure 5. Amide conformational shifts (ACS) and temperature coefficients (ATC) of **2**: (***A***) in water, (***B***) in 10% H_2O/90% CH_3OH, (***C***) in water in the presence of 1 equiv of **1**, and (D) in 10% H_2O/90% CH_3OH in the presence of 1 equiv of **1**.

a well-defined coil−helix equilibrium with clearly defined hydrogen bond lengths. In our case, we observed some $i + 3$ periodicity consistent with the "helical pitch" of an amphiphilic helix resulting in a curved helix with longer hydrogen bonds on the convex side and shorter hydrogen bonds on the concave side.

To ensure the mode of binding was 1:1, a Job CD titration was conducted with peptide **2** and receptor **3**. Maximum signal change in the Job titration was observed when the molar composition of **2** and **3** were equivalent, indicative of the 1:1 complex (Figure 6). Additionally, NMR shows evidence for the formation of the complex from the view of both the peptide (see below and ref 6) and the receptor. The guanidinium protons at 7.46, 7.51, and 7.61 ppm were observable for the receptor **1** alone, but not detectable in the presence of peptide **2**. Addition of >1 equiv of receptor **1** results in a reappearance of these guanidinium protons. This result suggests, in addition to the observation[6] of strong diastereotopic splitting of all Asp β-protons and the high chemical shift dispersion of the corresponding Asp amide protons (0.7 ppm in the presence of **1** and only 0.1 ppm in its absence), a specific interaction of the Asp residues with the complementary guanidinium ions on the receptor.

Control experiments were designed to show the significance of the charge interactions between the tetraguanidinium and tetraaspartate groups. The role of the bidentate guanidinium charge interactions was shown in a second control titration experiment of **2** with tetracationic spermine. Spermine has been shown to enhance the helical content of a dodecamer peptide containing four glutamic acid residues in an i, $i + 4$, $i + 7$, $i +$ 11 arrangement from 19% to 38% helicity.[3b] In our case, no increase of peptide helicity was observed after addition of equimolar amounts of spermine (**4**) (Figure 2, Table 1). On the contrary, addition of spermine provokes a small red shift and enhancement of the first minimum from 208 to 206 nm. Calculation of the "*R*-factor", defined as the quotient of the absorption at 222/208 nm and close to 1.0 for highly helical

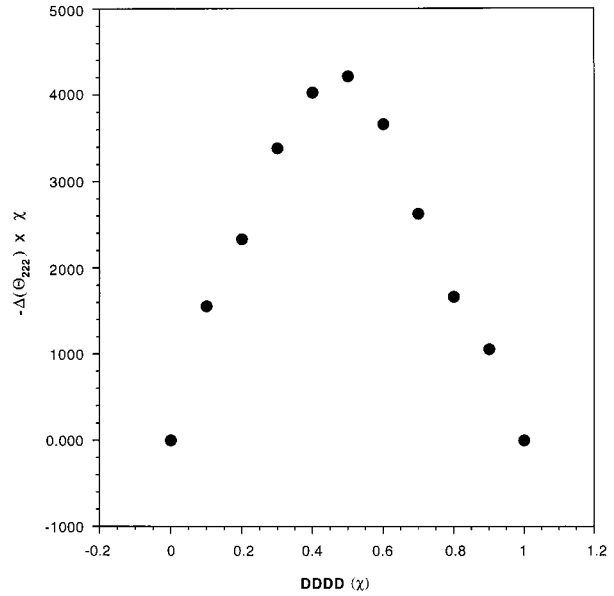

Figure 6. CD Job titration between DDDD (**2**) and tetraguanidinium receptor (**3**) in 10% H_2O/90% CH_3OH. The maximum signal change is observed at 0.5 mol fraction **2**, indicative of a 1:1 stoichiometry.

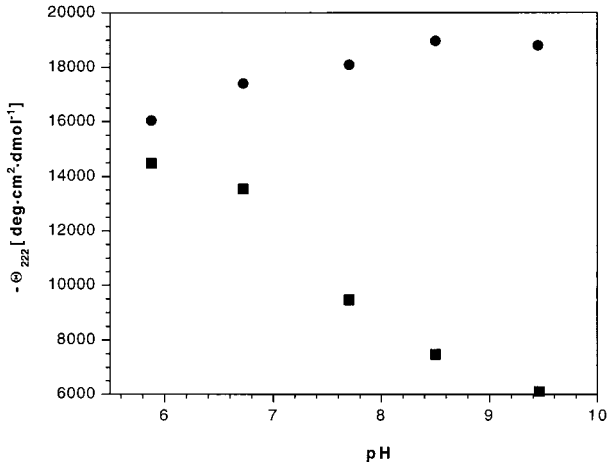

Figure 7. Ellipticity ($-\Theta_{222}$) values of peptide **2** at various pH values in the absence (■) or presence (●) of 2 equiv of receptor **1**. Buffers used were MES pH 6, HEPES pH 7 and 8, and CHES pH 9 and 10. Values plotted are uncorrected pH meter readings.

structures, gave 0.79 for the peptide in the absence of **1**, 0.83 in the presence of **1**, and 0.46 with spermine, respectively. Thus, the observed *R*-factors are consistent with a high helical content for the peptide in the presence of the receptor but indicate some destabilization of the helical structure and/or a stabilization of the unordered conformation in the presence of spermine as counterion.

The significance of pH for binding was also evaluated in this system by observing the CD signal of peptide **2** alone and in the presence of 2 equiv of **1** in 10 mM buffered aqueous methanol (Figure 7) at several pH values. The helicity of **2** decreases with increased pH, but is stabilized by complexation with receptor **1** at high and low pH values. The data suggest that at low pH the peptide becomes partially protonated, alleviating unfavorable intrastrand charge−charge interactions between aspartate side chains which increases the inherent helicity of the peptide. This change in the charge state of the peptide may also affect the nature of its solvation. Additionally, this partial protonation disfavors intermolecular interaction between the peptide carboxylates and receptor guanidiniums. The results indicate that the helical stabilization is due to specific interactions between **1** and **2**, and not pH changes as a result of added receptor. A titration of **2** with 2 equiv of **1** gave pH readings of 8.7 at the beginning and 8.2 at the end of the experiment. Binding titrations were carried out in 10 mM MES pH 6, CHES pH 9, or CHES pH 10 in 10% H_2O/90% (Table 1, Figure 8). Lower helical induction and affinity were observed in MES pH 6. The affinity and stabilization in the CHES buffer at pH 9 and 10 were similar to the purely aqueous system having K_a values of 1.9 and 1.8 $\times$ 10^5 M^{-1}, respectively, with stabilization of helicity being almost identical with the parent system.

Discussion

We have provided detailed information concerning the sequence specific recognition of an α-helical peptide by complementary charge interactions with an artificial receptor. The designed peptide contains four aspartic acids in an *i*, *i* + 3*n* (*n* = 0, 1, 2, 3) arrangement providing a negatively charged surface. The artificial receptor is a sterically restricted, tetrameric guanidinium derivative constructed by the stepwise dimerization and then tetramerization of bicyclic guanidinium subunits whose features will be discussed more in detail below.[5] Our spectroscopic results for peptide **2** alone show the absence of regular

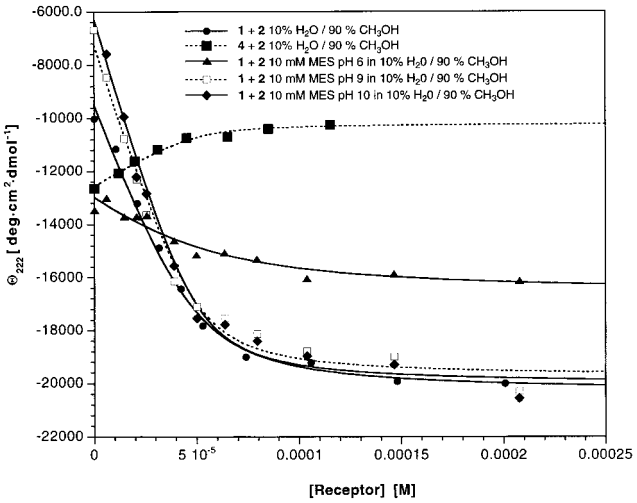

Figure 8. CD binding titrations of **2** with receptor **1** in (●) 10% H_2O/90% CH_3OH, (▲) 10 mM MES pH 6 in 10% H_2O/90% CH_3OH, (□) 10 mM CHES pH 9 in 10% H_2O/90% CH_3OH, and (◆) 10 mM CHES pH 10 in 10% H_2O/90% CH_3OH. Control CD binding titration of **2** with spermine (**4**) is also shown (■).

structure in water and the tendency to adopt an α-helical conformation (22% helical) in aqueous methanol. This helix is in a two-state equilibrium with the unordered state. Furthermore, detailed NMR studies show a more stable helical structure in the middle of the sequence and some conformational flexibility or end-fraying toward the N- and C-termini.[29] We have acquired evidence for the formation of an amphipathic helix with a hydrophilic side containing the acidic aspartate residues and a hydrophobic side containing alanine and leucine residues. From these results we are able to propose two different scenarios for the complexation of extended peptide sequences by our synthetic receptors.

In the first (water), all four negatively charged aspartate side chains are randomly exposed to both solvent and receptor in arbitrary orientations. The result is a large ensemble of conformations without any structural preference. In the second scenario (aqueous methanol), there is a significant population of a helical conformation in which the four critical residues are predisposed toward the receptor with a complementary orientation (Scheme 1). Consequently, simultaneous recognition of all four aspartate side chains in peptide **2** by the tetraguanidinium receptor **1** should be entropically preferred. Here the side chain functionality of **2** is better complemented by the receptor molecule **1**.

We have strong evidence for the formation of a 1:1 peptide−receptor complex in aqueous methanol by recognition of the complementary charges. Conclusive proof for this has been provided by the observation of intermolecular NOEs between protons of the side chains of the peptide and the most accessible protons of the tetraguanidinium receptor.

Our results compare well with those observed by Sasaki.[3b] The peptide sequence used in the Sasaki system contained glutamates in an *i*, *i* + 4 orientation. This spacing presents the carboxylate functionality linearly along one face of the α-helix. Helicity was doubled upon binding of receptor (spermine) with an association constant of 2 $\times$ 10^4 M^{-1} in 30% TFE buffer at 4 °C, which is comparable to our system. These results suggest that spermine effectively binds the peptide in an α-helical conformation when the carboxylates approach a linear presenta-

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tion along the helix surface. In our system the spacing (*i*, *i* + 3) of aspartates results in an arc or superhelix of functionality along the surface of the helix. We found that the tetraguanidinium series was able to recognize and stabilize the helix effectively, while spermine was not. Combined, these results underscore the importance not only of functional group complementarity in helix recognition, but the spatial disposition of this complementarity with respect to the primary and secondary sequence.

The investigation of complex formation in aqueous methanol assists in understanding the situation observed in water. We are not able to quantify helix stabilization in water, but we have evidence from NOE data for the formation of higher structural organization upon addition of the receptor. Thus, in water, the guanidinium receptor may be able to recognize the peptide but appears unable to induce significant helical structure.

From this we conclude that tetraguanidinium derivative **1** recognizes not only the primary sequence of a peptide, but also the resulting functional group disposition in an α-helical secondary structure. We have previously shown[6] the tendency of our artificial receptor to adopt a superhelical conformation in the presence of di- or tetravalent anions matching nearly perfectly with the tetraaspartate peptide **2** organized in an α-helical conformation. The receptor was designed to complement the Asp side chains in a superhelical array of functionality along the surface of the helix. We are now investigating natural proteins and peptides with this sequence motif (D/E-XX-D/E-XX-D/E-XX-D/E where X is any amino acid) located in (a) a helical conformation and (b) in an unordered loop. This should allow us to probe the ability of the artificial receptor to recognize protein sequence motifs containing anionic amino acids in different conformations on a protein surface.

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