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Simple Cation−π Interaction between a Phenyl Ring and a Protonated Amine Stabilizes an α-Helix in Water

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Abstract: Cation−π interactions have been proposed to be important contributors to protein structure and function. In particular, these interactions have been suggested to provide significant stability at the solvent-exposed surface of a protein. We have investigated the magnitude of cation−π interactions between phenylalanine (Phe) and lysine (Lys), ornithine (Orn), and diaminobutanoic acid (Dab) in the context of an α-helix and have found that only the Phe···Orn interaction provides significant stability to the helix, stabilizing it by −0.4 kcal/mol. This interaction energy is in the same range as a salt bridge in an α-helix, and equivalent to the recently reported Trp···Arg interaction in an α-helix, despite the fact that Trp···guanidinium interactions have been proposed to be stronger than Phe···ammonium interactions. These results indicate that even the simplest cation−π interaction can provide significant stability to protein structure and demonstrate the subtle factors that can influence the observed interaction energies in designed systems.

Introduction

The unique structure of a protein is defined by a large number of weak interactions that provide both stability and specificity to the folded structure. In addition to the traditional noncovalent interactions such as hydrogen bonds, hydrophobic interactions, and salt bridges, cation−π interactions between aromatic rings and cations have been proposed to play an important role in biological systems,[1] including protein structure,[2] stability of thermophilic proteins,[3] protein−ligand interactions,[4] and ion channels.[5] Extensive theoretical work,[6] gas-phase studies,[7] and investigations in host−guest systems[8] have been performed on these interactions, indicating that they are a significant force in molecular recognition. Mutation studies in proteins[2b,c] and statistical analyses of protein structures[9] have demonstrated their contribution to biomolecular structure and function. However, only recently have cation−π interactions been investigated in peptide model systems.[10] These systems have the advantage that the interaction can be studied in a well-defined environment in the absence of tertiary interactions. Furthermore, energies of different types of noncovalent interactions can be directly compared in these systems, providing a valuable method for elucidating their contribution to protein structure.

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The magnitude of the cation−π interaction is proposed to depend on the nature of both the aromatic and cationic groups involved. In the context of a protein, cation−π interactions can occur between Phe, Tyr, or Trp and Lys, Arg, or His. Of the aromatic residues, Trp has been shown to be the most common aromatic residue to participate in cation−π interactions,[8a] and theoretical studies indicate that it can form the strongest interaction with cations.[5a] This is believed to be due to the greater electron density of the six-membered ring on Trp relative to Tyr or Phe. Theoretical studies suggest that the interaction of a protonated amine with an indole ring is approximately 7−8 kcal mol^{-1} more favorable than the corresponding interaction with a phenyl ring in the gas phase.[1a]

Of the cationic amino acids, only Arg and Lys have been well-studied in the context of cation−π interaction in proteins.[8] In statistical analyses of protein structures, Arg was found to be more likely to form cation−π interactions than Lys.[8a] This is believed to be due in part to differences in the geometries of interaction between an aromatic ring and Arg or Lys. Arg preferentially interacts with an aromatic ring in a stacked geometry, hence increasing van der Waals interactions. This geometry may also allow the NH groups to hydrogen-bond, thus decreasing the desolvation penalty for interacting with an aromatic ring. The interaction between Lys and an aromatic ring does not involve the additional van der Waals interactions and must pay a larger desolvation cost, such that the interaction of an ammonium with an aromatic ring is weaker than that of a guanidinium with an aromatic ring.[9a] Thus, the interaction of a phenyl ring with a protonated amine can be considered the simplest type of cation−π interaction and is predicted to be the weakest in a protein.

A statistical analysis of protein structures has shown that approximately 60% of cation−π interactions in proteins are at least partially solvent-exposed.[8a] Moreover, a recent theoretical study suggested that cation−π interactions are expected to be more stabilizing than a salt bridge at a solvent-exposed surface of a protein since only one of the two interacting residues has a significant desolvation cost.[5a] These studies suggest that cation−π interactions play an important role in stabilizing protein structure at the surface. However, a recent investigation of a model cation−π interaction between Phe and Arg in an α-helical peptide showed no increase in helicity.[9a] Only the Trp···Arg pair was found to stabilize an α-helix.[9b] We had undertaken a similar study of the influence of cation−π interaction on stability of an α-helix, and we have found that even the simplest cation−π interaction between a phenyl ring and a protonated amine can stabilize an α-helix as much as a salt bridge.

Experimental Procedures

Peptide Synthesis and Purification. Synthesis of the peptides was carried out by manual solid-phase synthesis or on an automated Pioneer peptide synthesizer. Peptides synthesized by manual solid-phase synthesis were attached to a Rink amide resin. The 9-fluorenylmethoxycarbonyl (Fmoc) group was used for temporary protection of the α-amino group, and *tert*-butyl groups were used for all the side-chain functionalities. Fmoc deprotections were performed by treatment with 20% piperidine/*N*,*N*-dimethylformamide (DMF) for 30 min at room temperature. To incorporate the first amino acid residue, 8 equiv each of Fmoc amino acid, 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU), and *N*-hydroxybenzotriazole (HOBt) were dissolved with 16 equiv of diisopropylethylamine (DIPEA) in DMF. Sequential couplings used 2.5 equiv of Fmoc amino acid, HBTU, and HOBt with 5 equiv of DIPEA. Coupling reactions were run for 3−6 h. The Rink resin was washed with DMF, methanol, and methylene chloride. The coupling efficiency for each step was monitored by the Kaiser test.[11] After a positive (blue) Kaiser test, recoupling was performed. Peptides synthesized on a Pioneer peptide synthesizer were carried out on Fmoc-PAL-PEG-PS resin (void volume 4.4 mL/g) on a 0.06 mmol scale by the continuous flow method. Couplings used 4 equiv of Fmoc amino acid, 4.5 equiv of HBTU and HOBt, and 14 equiv of DIPEA in DMF. Standard coupling cycles (45 min) were used for the first 7−9 amino acids, and extended couplings (75 min) were used to complete the sequence. Fmoc deprotection was carried out in 20% piperidine in DMF for 10−60 min. After completion of the sequence, the N-terminus was acetylated with acetic anhydride/DIPEA/DMF (1:2:7) for 1 h.

Peptides were cleaved from the resin with simultaneous side-chain deprotection by treatment with a mixture of 96% trifluoroacetic acid (TFA), 2% H_2O, and 2% triisopropylsilane (TIPS) for 3−4 h at room temperature. After filtration, TFA was evaporated and cleavage products were precipitated with diethyl ether, and the water-soluble peptides were extracted twice with water. Crude peptides were obtained after lyophilization.

Peptides were purified by reverse-phase HPLC. Samples were prepared by dissolving the peptides in a mixture of 98.9% H_2O, 1% acetonitrile, and 0.1% TFA. Samples were then monitored at 220 and 280 nm with a Waters 600 solvent delivery system and a Waters 486 variable-wavelength detector. A semipreparative Vydac C-18 column was used with a gradient of eluent A (95:5 water/acetonitrile) to eluent B (95:5 acetonitrile/water), flow rate 4 mL/min, linear gradient elution from 100% A and 0% B to 40% A and 60% B over 50 min. Both solvents contained 0.1% TFA. The identity of each peptide was confirmed by matrix-assisted laser desorption ionization (MALDI) mass spectrometry and amino acid analysis.

Circular Dichroic Measurements. Stock solutions of the purified peptides were prepared by dissolving them in 10 mM sodium phoshate and 0.1 or 1.0 M sodium chloride buffer, pH 7.5. The concentration of each peptide in 5 M guanidinium chloride was then determined from the absorbance of the Tyr residue at 275 nm (ϵ = 1450 M^{-1} cm^{-1}). CD spectra were acquired on an Aviv 60DS CD spectrometer, using a quartz window cell of 1 mm optical path length. All scans were taken at 0−1 °C from 190 to 250 nm. Helical contents were determined from the ellipticity at 222 nm, $[\theta]_{222,obs}$, and were calculated according to

$$f_H = ([\theta]_{222,obs} - [\theta]_{222,0}) / ([\theta]_{222,100} - [\theta]_{222,0}) \qquad (1)$$

where f_H is the fraction helix. The values used for $[\theta]_{222}$ at 0% and 100% helicity, $[\theta]_{222,0}$ and $[\theta]_{222,100}$, were 0 and $-40\,000(1 - 2.5/n)$ deg cm^2/dmol, respectively, where n is the number of residues in the peptide.[12]

Concentration Study. A concentration dependence study was performed on F8K12 to confirm that the peptides did not aggregate under the conditions studied. Solutions of F8K12 in buffer were prepared at eight different concentrations between 50 and 125 μM. $[\theta]_{222}$ was determined for each solution by CD and was found to be invariant in this concentration range.

Quantification of Side Chain−Side Chain Interactions. The measured helix contents were analyzed by AGADIR helix−coil theory for both the i, $i + 4$ and i, $i + 5$ peptides.[13] First the helix content was determined by AGADIR theory, with the i, $i + 4$ or i, $i + 5$ interaction

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(a) **F8X12**: Ac-AAKAAKA-**F**AAA**X**AK-AAKK-GGY-NH_2

(b) **F7X12**: Ac-AKAAKA**F**-AAAA**X**AK-AAKK-GGY-NH_2

A = Ala, alanine; K = Lys, lysine

X = K, O (Orn, ornithine), or Dab (diaminobutanoic acid)

(c)

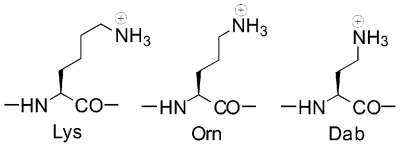

Figure 1. Peptide sequences under investigation: (a) *i*, *i* + 4 sequence; (b) *i*, *i* + 5 sequence; (c) amino acid structures of residue X.

energies set to zero; then the interaction energies were varied until the calculated helix content values matched those experimentally obtained. The helicities of the *i*, *i* + 5 peptides as determined by AGADIR were shown to correspond with the experimentally determined helicities.

NMR Measurements. NMR samples were made by dissolving 3−6 mg of peptide into 650 μL of a solution of 30% methanol-d_4 in sodium acetate-buffered D_2O, pH 3.8 (uncorrected), with DSS as an internal standard. All pulse sequences are used directly from Varian's Chempack. Total correlation spectroscopy (TOCSY) and double-quantum correlated spectroscopy (DQCOSY) are used to make general proton assignments. Unambiguous chemical assignments cannot be made for the entire helix, but due to the unique chemical shifts of the aromatic residue and *i* + 4 residues, clear assignments could be made. The nuclear Overhauser effect spectroscopy (NOESY) spectrum is taken with 16−64 scans in the direct dimension with 256−512 increments in the indirect dimension. The mixing time for the NOESY spectra is either 100 or 200 ms. Presaturation of 1 s is used in the direct dimension with a saturation power of 2. The spectrum is optimized by use of standard window functions (Gaussian or sine bell with shifting). Linear prediction is used in all spectra and never exceeds the number of increments.

Results

Peptide Design. Initially we investigated the interaction of Phe with Lys at the *i*, *i* + *4* and *i*, *i* + *5* (control) positions of an α-helical Ala-Lys peptide (Figure 1).[14] The host sequence was chosen to give high helicities and water solubility. The lysines were spaced such that only Lys12 could interact with Phe in the *i*, *i* + 4 spacing. The two side chains at positions 8 and 12 in peptide F8K12 are in close proximity on the same side of the helix, whereas in the control peptide F7K12 with Phe and Lys spaced *i*, *i* + 5, the residues are on opposite sides of the helix and too far apart to interact.

Circular Dichroism. The helical structure of each peptide was determined by circular dichroism (CD) and the helicity was calculated from the mean residue ellipticity at 222 nm. As with the previously reported study of Phe···Arg interactions in α-helices,[10b] we found no significant difference in helicity between F8K12 and F7K12, suggesting that cation−π interactions do not stabilize a monomeric α-helix (Figure 2a). Because a significant entropic cost is expected in the interaction of Lys with Phe, we investigated the influence of the side-chain length on the ability of a cation−π interaction to influence α-helix stability.

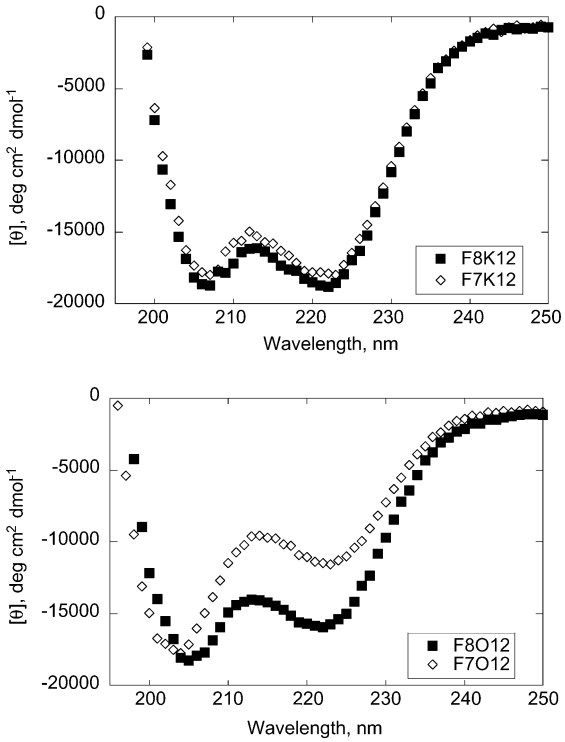

Figure 2. Circular dichroic spectra of (a) F8K12 (9) and F7K12 (]) and (b) F8O12 (b) and F7O12 (]). All spectra were measured at 0−1 °C, 80 mM peptide, 0.1 M NaCl and 10 mM phosphate buffer, pH 7.5.

Table 1. Mean Residue Ellipticities, Helicities, and Side Chain−Side Chain Interaction Energies of Peptides[a]

peptide	$[\theta]_{222}$[a] (deg cm^2 dmol^{-1})	% helix[b] (±3%)	$\Delta\Delta G^c$ (kcal/mol)
F8K12	−18 800	52	0
F7K12	−17 900	49	0
F8O12	−15 900	43	−0.4
F7O12	−11 500	32	0
F8Dab12	−11 600	34	ND[d]
F7Dab12	−9 900	29	ND[d]

[a] Conditions: 0−1 °C, 0.1 M NaCl, 10 mM phosphate buffer, pH 7.5. [b] Determined from the mean residue ellipticity as described in the Experimental Section. [c] $\Delta G°$ is the interaction energy between Phe and Lys or Orn. It was determined with AGADIR, by allowing the interaction energy to vary until the calculated helicity fit the observed helicity. The helix propensity for Orn was taken from ref 12. [d] The interaction energies in F8Dab12 and F7Dab12 were not determined.

The *i*, *i* + 4 interactions of Phe with ornithine (Orn), which has one fewer methylene units than Lys, and diaminobutanoic acid (Dab), which is two methylene units shorter than Lys, were studied by CD and compared to their *i*, *i* + 5 controls (Figure 1). Both Orn and Dab have lower helix propensities, and so the overall helicity of the peptides containing these residues was lower than for peptides with Lys for both *i*, *i* + 4 and *i*, *i* + 5 peptides.[12] Inspection of F8Dab12 indicates that it is not appreciably more helical than its *i*, *i* + *5* control peptide, as was found with F8K12 (Table 1). In contrast, F8O12 and F7O12 have significantly different helicities (Figure 2b). The increased helicity of F8O12 relative to F7O12 suggests the presence of a cation−π interaction between Phe and Orn. By use of the AGADIR helix−coil transition model to fit the CD data, the magnitude of the Phe···Orn side chain interaction was found to be −0.4 kcal/mol.[12]

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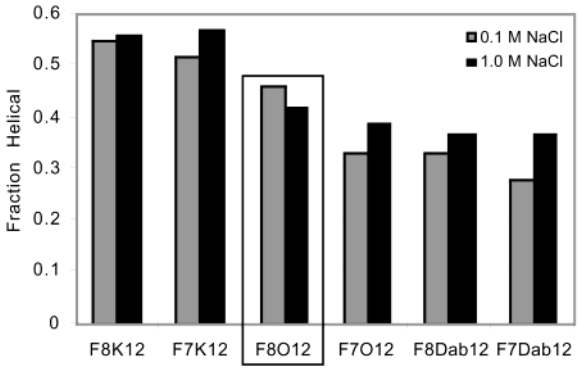

Figure 3. Mean residue ellipticities of peptides at 0.1 M NaCl (gray bars) and 1.0 M NaCl (black bars), 0−1 °C, 10 mM phosphate buffer, pH 7.5.

Salt Titration. Salt concentration studies provide further evidence for the presence of a cation−π interaction in F8O12 (Figure 3). Helicities for each of the peptides were determined at 0.1 and 1.0 M NaCl. With the exception of F8O12, all the peptides studied increase in helicity at higher salt concentrations, as expected.[16] However, F8O12 is less helical at higher salt concentrations, which is consistent with shielding of the cation−π interaction. It is worth noting that both F8K12 and F8Dab12 show smaller increases in helix stability at high salt concentrations than their *i*, *i* + 5 counterparts, suggesting that there may be some small contribution of cation−π interaction in each case.

NMR Study. We have also investigated each *i*, *i* + 4 peptide by NOESY NMR in 30% MeOD/D_2O. The *i* + 4 charged residues were easily identifiable in the NMR spectra because the methylene hydrogens neighboring the ammonium group were all upfield-shifted relative to the other lysine residues in the peptide. This is presumably due to ring current effects resulting from the close proximity of Phe. In 30% MeOD/D_2O at 25 °C, only an NOE between Phe and Orn was observed (Figure 4).[17] When the solution is cooled to 5 °C, a strong NOE was observed between Phe and Orn, and a weak NOE was observed between Phe and Dab as well as between Phe and Lys. These data provide further support for the presence of a cation−π interaction between Phe and Orn and again suggest that there may also be a weak cation−π interaction in both F8K12 and F8Dab12.

Discussion

We have found that a Phe···Orn cation−π interaction stabilizes an α-helix by −0.4 kcal/mol, which is equivalent in magnitude to a recently reported Trp···Arg interaction in an α-helix.[10b] This was surprising since both experimental and theoretical work indicate that the Trp···Arg interaction is expected to be more favorable than the interaction of an ammonium ion with Phe.[5f,8a,9a,9f] Moreover, no measurable stabilization of an α-helix was found for Phe···Arg,[10a] Phe···Lys, or Phe···Dab. The magnitude of the observed interaction may be influenced by a number of different factors, including differences in conformational entropy, hydrophobic and van der Waals interactions, side chain−helix barrel interactions, and rotamer populations. Thus, the observed interaction energies likely reflect the balance between maximizing the cation−π interaction with the loss of other favorable interactions and conformations. It is interesting to note that a study by Kemp et al. in which Lys was compared to norleucine (Nle) in an alanine helix showed that Lys is significantly more helix-stabilizing than Nle, but in the comparison of Orn to norvaline (Nve), very little difference was observed. This was interpreted as indicating that Lys has a unique method of stabilization in an alanine helix through optimal charge−dipole interactions between Lys and the helix barrel. If this is the case, a cation−π interaction between Phe and Lys may not have been observed because the interaction is energetically similar to the interaction of Lys with the helix barrel. Kemp's work suggests that this side chain−helix barrel interaction is negligible for Orn, which correlates well with our finding that the Phe···Orn interaction measurably stabilizes the helix.

Within the context of protein structures, the ϵ-CH_2, rather than the ammonium group of Lys, is often observed to interact with an aromatic ring.[9a] This methylene has a significant partial positive charge and thus may interact with the aromatic ring via a cation−π interaction while allowing the ammonium group to be solvated. Although we cannot differentiate between a cation−π interaction between Phe and the protonated amine of Orn or the δ-methylene of Orn, we observe an NOE between Phe and the δ-methylene group. Weak NOEs are also seen between the ϵ-methylene of Lys and Phe and the γ-methylene of Dab and Phe at low temperatures. This suggests that, in all three cases, there is an interaction between the methylene neighboring the ammonium group and the aromatic ring. The fact that some screening of the interaction is observed at high salt concentrations for all three peptides supports the fact that there is an electrostatic component to the interaction, such that it cannot be described as strictly due to hydrophobic or van der Waals interactions. The observed differences between Lys, Orn, and Dab are thus best attributed to different conformational preferences and competing interactions rather than differences in the cation−π interaction itself.

Salt bridges have been shown to contribute from 0 to −0.75 kcal/mol to the stability of an α-helix.[18] Thus, our studies indicate that even a simple cation−π interaction between a

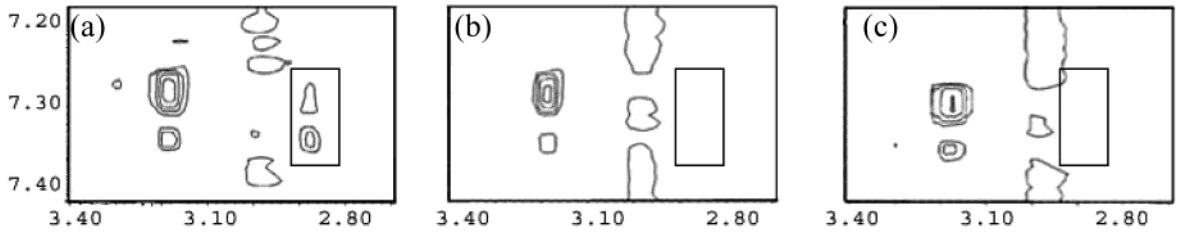

Figure 4. Normalized NOESY spectra for (a) F8O12, (b) F8K12, and (c) F8Dab12 at 25 °C in 30% MeOD/D_2O. The leftmost peak in each spectrum is the NOE cross-peak between the phenylalanine aryl protons and the phenylalanine β-protons and is shown for reference. The box indicates the expected location of the NOE between the phenylalanine aryl protons and the Lys, Orn, or Dab side chain. The peaks at approximately 3 ppm are positive projections and represent noise.

phenyl ring and a protonated amine, which is expected to be the weakest cation−π interaction in proteins, can contribute at least as much as a salt bridge at a solvent-exposed surface of an α-helix, in agreement with theory.[5a] However, it is important to note that the magnitude of both the cation−π interactions and the salt bridges are lower than predicted, likely due to other competing forces.

Conclusions

We have found that a simple phenyl···ammonium cation−π interaction can provide stability to an α-helical peptide in aqueous solution. The fact that the magnitude of the interaction between Phe and Orn is equivalent to that of Trp···Arg interaction in an α-helix suggests that the observed interaction energy is influenced by subtle differences in the factors contributing to helix stability, such as conformational preferences, entropic costs, and interactions with the helix backbone, rather than indicating that a phenyl···ammonium interaction is as strong as an indole···guanidinium interaction. In addition, we have shown that the magnitude of a phenyl-ammonium interaction is similar to a salt bridge in an α-helix in aqueous solution. Thus, these studies provide evidence that simple cation−π interactions can indeed be important contributors to the stability of a protein at its surface.

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(16) Higher salt concentrations screen the helix macrodipole as well as any repulsive interactions between the Lys residues.
(17) In D_2O at 5 °C no NOEs were observed between Phe and Lys, Orn, or Dab.
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