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Non-classical helix-stabilizing interactions: C–H···O H-bonding between Phe and Glu side chains in α-helical peptides☆

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Abstract

The classical picture of H-bonds has evolved considerably. In contrast to earlier expectations, C–H···O H-bonds are now known to be prevalent in both small organic and large biological systems. However, there are few reports on the energetic contribution of C–H···O H-bonds in protein or polypeptide systems and we do not know whether such interactions are stabilizing. Here we investigate C–H···O H-bonding interactions between Phe and Glu side chains by determining their effects on the helicity of model α-helical peptides using a combination of CD and NMR spectroscopy. The results suggest that Glu/Phe C–H···O H-bonding interactions stabilize helical structure, but only in the orientation Glu→Phe (N→C). Each Glu→Phe (N→C) interaction can contribute approximately −0.5 kcal mol^{-1} to the stability of helical peptide. In the reverse orientation, Phe→Glu (N→C) appears to contribute negligibly. pH titrations provide further evidence for the existence of C–H···O H-bonds. The C–H···O H-bonding interactions in these peptides are insensitive to the screening effect of added neutral salt. Our results provide quantitative energetic information on C–H···O H-bonds that should be useful for empirical force-field calibration.

Keywords: C–H···O H-bonds; Free energy; Energetics; α-Helix

1. Introduction

In two papers published in 1955, John Schellman initiated an approach to understanding the stabilization of α-helical and β-sheet structures that is remarkably current today [1,2]. First, he used data on the dimerization of urea to estimate the free energy contribution of the H-bond between N–H and O=C of urea in water. His conclusion was that the enthalpy of an N–H···O=C H-bond in water is −1.5 kcal mol^{-1}, stabilizing relative to the competing bonds from water itself. Estimates of N–H···O=C H-bond stability from titration and calorimetry experiments are not far from this value [3–5]. In the second paper, he showed that α-helical structure should have a critical size of between 15 and 20 residues, again in agreement

☆ This article is respectfully dedicated to Professor John A. Schellman, whose pioneering work on helix stabilization in water has influenced theoretical and experimental advances in the field for nearly 50 years.

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with recent studies. He also presented a treatment of the effects of temperature and urea on secondary structure. Since that time, several groups have attempted to define the full set of interactions that stabilize helical structure. The first approach, due to Scheraga [6], was based on host–guest polypeptides, in which natural side chains were copolymerized with a host side chain, and the effect on helix content determined. A second focused on short, soluble peptides, a program that received its initial impetus from the discovery by Marqusee et al. that short, alanine-rich peptides form moderately stable helical structures in aqueous solutions [7]. Building on this foundation, it became possible to determine the nucleation constant, propagation constants and the effects of a large number of interactions on the stability of helical structure; side chain–chain interaction and helix capping at the N and C termini have all been quantitatively studied [8–20].

Nevertheless, the results to date remain quantitatively inadequate: predictions by the AGADIR algorithm, for example, do not quantitatively reproduce the helix content of many peptides [21,22]. One reason for this situation is that the library of component interactions between pairs of side chains spaced k residues apart $(1<k<4)$ is very large, and has not been exhaustively mapped out to date. A second is the potential existence of non-additive cooperative effects between pairs of residues that interact with each other, which would imply that interactions beyond pairs of residues must be included in the library. We evaluate here a new side-chain–side-chain interaction between aromatic and acidic side chains that involves C–H···O H-bonds, which appear to contribute significantly to helix stability. Together with recent data on cation–π interactions [20,81], this suggests that a comprehensive description of side-chain–side-chain interactions will entail analysis of a number of weak interactions that have not been systematically evaluated so far.

Hydrophobic forces and/or H-bonds are currently viewed as the main driving force for protein folding in general [23]. Van der Waals' interactions appear to play a more important role in current thinking—the idea that protein cores are energetically interchangeable arose from experiments that did not assess the differences in energies. In any case, these ideas provide a qualitative explanation of protein structure, folding and stability. The fundamental folding question will not be solved until a more elaborate quantitative description of all the physical forces that govern protein structure, stability and folding is available. The role in biochemistry of non-conventional non-covalent interactions, including cation–π interactions [24,25], C–H···O and other non-classical H-bonds is being recognized [26,27]. In addition, the classical picture of H-bonds has evolved considerably. The impact for proteins is that a number of C–H groups, along with the previously accepted N–H and O–H groups, can serve as donor groups, while in addition to N and O, π-systems can also serve as acceptor groups [28].

The existence of C–H···O hydrogen bonds was first suggested more than 60 years ago [29,30]. Pauling noted the fact that the boiling point of acetyl chloride is 51 °C higher than that of trifluoroacetyl chloride and suggested that this increase could be the result of C–H···O H-bonds [31]. However, when Sutor proposed the existence of this general type of H-bonding in the early 1960s [32,33], her suggestion was greeted with skepticism. Theoretical studies by Kollman et al. [34] supported Sutor's claim, as did other early proposals for C–H···O contacts [35–37]. Neutron diffraction studies on small molecules by Taylor and Kennard finally presented unequivocal evidence for the existence of this type of H-bond by 1982 [38]. C–H···O H-bonds are now understood to be present in many biological systems, including proteins [27,39–42], nucleic acids [43–49] and carbohydrates [50]. C–H···O H-bonds have also been implicated in the interaction of nucleic acids with proteins [51,52], drug binding [53–55], the stability and specificity of transmembrane helices [56], the mechanism of serine protease catalysis [26,57], crystal engineering [58–60] and supermolecular assemblies [61].

The $C_\alpha H$ group of each amino acid residue in a protein is directly adjacent to a pair of electronegative groups, and hence serves as a relatively stronger proton donor compared to other $C(sp^3)$–

Table 1
The sequences of peptides used in this study

Peptide	Sequence
EFE4-4	Ac–OOAAAAAEAAAFAAAEAAAAAOOY–NH_2
EFE5-5	Ac–OOAAAAEAAAAFAAAAEAAAAOOY–NH_2

Ac, acetyl; O, ornithine; A, alanine; E, glutamic acid; F, phenylalanine; Y, tyrosine.

H groups that lack any substitution from electronegative groups. It is among the most prevalent CH group involving the C–H···O interaction, and therefore many investigations of C–H···O H-bonds focus on the C_α–H groups as donor [39,40,62–66]. Other weakly acidic CH groups, including aromatic CH groups, the C_δH of proline, the C_εH of histidine and the C_εH of lysine, should also be able to participate in C–H···O H-bonding interactions. There are few experimental studies [20] of the energetic contribution from any type of C–H···O H-bonds in protein or polypeptide systems, although there are a number of theoretical estimations of the strength of C_α–H···O H-bonds from real amino-acid residues [66] or model organic compounds [62–65].

Peptide models have played a major role in the effort to define the contribution of specific amino-acid side-chain interactions to stabilizing helical structures. In peptides the background can be precisely specified. Moreover, the effects of weak interactions between side chains can be readily detected as shifts in conformational equilibrium by CD or NMR, and quantitative free-energy values can be assigned by fitting CD spectral data to appropriate multi-state transition models. Peptides have been extensively used to evaluate the interaction free energies between pairs of side chains in a helix; examples include hydrogen bonds [8–10], salt bridges [11–13], aromatic and hydrophobic interactions [14–16], helix-capping [17–19], cation–π interactions [20,81], etc. Here we use a model α-helical alanine peptide system to evaluate the energetics of solvent exposed C–H···O interactions on the surface of helices. We study two alanine-based peptides containing Glu and Phe residues located at $(i,i+4)$ or $(i,i+5)$ positions (Table 1). After fitting CD data on the models using helix–coil transition theory, we conclude that the C–H···O interaction between Phe and Glu side chains contributes favorably to helix stability. Combined with our previous studies [20], the results suggest that Glu/Phe C–H···O H-bonding interactions stabilize helical structure, but only in the orientation Glu→Phe (N→C). Each Glu→Phe (N→C) interaction can contribute approximately -0.5 kcal mol^{-1} to the stability of helical peptide. The reverse orientation Phe→Glu (N→C) appears to contribute negligibly.

2. Materials and methods

2.1. Peptide synthesis and purification

Peptides were synthesized by solid-phase peptide synthesis on a Rainin PS3 automated synthesizer using Rink resin (Advanced Chemtech) and Fmoc chemistry. Cleavage from the resin and removal of side-chain protecting groups was performed with 90% TFA in the presence of the scavenger anisole and H_2O. Crude peptides were precipitated in cold ether, dissolved in water and lyophilized. Purification was performed by HPLC on a Delta Pak C18 reverse-phase semi-preparative column. Molecular masses were confirmed by MALDI mass spectrometry using a Kratos MALDI I linear time-of-flight spectrometer.

2.2. CD measurements

Stock solution concentrations were determined by tyrosine absorbance in 6 M guanidine HCl ($\varepsilon_{275}=1450$ M^{-1} cm^{-1}) [67]. Stock solutions were prepared in 10 mM phosphate buffer (pH 7.0) at a concentration of 500–1500 μM. CD measurements were performed at a peptide concentration of 50 μM in 10 mM phosphate buffer (pH 7) at 4 °C unless otherwise specified. Concentration-dependent CD measurements were conducted over a range of peptide concentrations from 10 to 400 μM. Salt concentration variations were performed by preparing a 3 M NaCl solution in 10 mM phosphate buffer and diluting to 0.5, 1.0 and 2.5 M NaCl. pH experiments were performed by diluting a stock solution of peptide in citrate–phosphate–borate buffers prepared at different pH values from 2.0 to 13.0. CD measurements were

recorded on an Aviv DS 60 CD spectrometer equipped with a temperature controller. The helix content of each peptide was determined from the mean residue CD at 222 nm, $[\theta]_{222}$ (deg cm^2 dmol^{-1}) corrected for the length of the chains according to Manning and Woody [68]. The wavelength of the instrument was calibrated using (+)-10-camphorsulfonic acid [69].

2.3. CD data analysis

Analysis of the free energy contribution of side-chain–side-chain interaction from CD data was carried out using a modified Zimm–Bragg multistate helix–coil transition model previously described [70,71]. The relation between fraction helicity (f) and CD is taken as $f=[\theta]_{222}/[\theta]^{o}_{222}$, where $[\theta]^{o}_{222}$ is the estimated molar residue CD signal at 222 nm, $-34\,000$ for an α-helix of 24 residues [71].

In addition to the nucleation constant σ and a set of helix propagation constants s_i corresponding to each species of amino acid (i) in the sequence, the model explicitly introduces an additional interaction constant $\gamma=\exp(-\Delta G/RT)$, where ΔG refers to the interaction between side chains. The weighting for a chain of N residues is recursively generated from the weights of shorter chains using difference equations described by Gans et al. [70] and Yang et al. [71]. Side-chain–side-chain interactions spaced at $(i,i+4)$ are weighted by the additional stability constant γ. The nucleation constant is assumed to be independent of sequence, with a value of 0.004 [71]. Intrinsic helix propensities at 4 °C were taken to be sAla = 1.5, sGlu = 0.43, sOrn = 0.53, sPhe = 0.33 and sTyr = 0.45 [72,73]. We fitted the CD data to the helix–coil transition model for individual peptides [11,70].

2.4. NMR spectroscopy

^{1}H NMR spectra were collected on a Varian UNITY 500 spectrometer. We used the States method [74] to obtain phase-sensitive clean TOCSY [75,76] spectra using a mixing time of 80 ms. NOESY experiments [77,78] were run with a mixing time of 400 ms. Water suppression was achieved using a Watergate sequence [79]. Each 2D data set contained 512 FIDs with 2048 complex data points each, obtained by collecting 64 added free induction decays after four dummy scans. Spectra were Fourier transformed in both $t2$ and $t1$ dimensions after apodization with a shifted square-sine bell function, typically with an 80° phase shift. Zero filling was carried out in the $t1$ dimension to obtain a final matrix of 2048×1024 real points. NMR data were processed using VNMR (version 6.1A). Samples were prepared by dissolving peptides in 10 mM phosphate buffer (pH 7, 10% D_2O) to a concentration of ca. 5 mM. The sodium salt of 3-(trimethysilyl)-[3,3,2,2-^{2}H]propionic acid was used as an internal chemical shift reference.

3. Results

3.1. Peptide design

As demonstrated in several previous studies [13,20,80,81] on model alanine helical peptides, the high helix-forming propensity of alanine allows short peptides to form helical structure in water while reducing the extent of side chain interference. Our strategy positions ornithine residues outside the range of the target interactions to provide solubility and prevent aggregation. Strict conservation of composition within these two peptides avoids differences in intrinsic helical propensity in each chain. Differences in helicity between two peptides thus reflect positional effects and/or side-chain–side-chain interactions. This strategy avoids differences in reference states that can complicate evaluating the interactions specified.

The peptides of this study are 24-mers with a Tyr residue in each peptide serving to determine concentration (Table 1). EFE4-4 was designed to allow quantitative evaluation of the role of Glu/Phe C–H···O interactions on helix stability without the interference from a potential cation–π interaction between Arg and Phe as in our previous study on EFR peptides [20,81]. EFE5-5 was designed as a control for the peptide EFE4-4. The arrangement in EFE5-5 places Glu and Phe side chains at sites on non-contiguous faces of a helix and far apart. In these two peptides, we shift the position of Glu while keeping the Phe residue in

the same center position. The EFE4-4 peptide designed can also be used to investigate the effect of orientation on the interaction at the same time by comparing the results to our previous study of EFR peptides [20].

3.2. CD analysis

The CD spectra (Fig. 1) show that both peptides are helical, with characteristic minima at 222 and 208 nm and an iso-dichroic point near 202 nm, consistent with a monomeric two-state helix–coil transition. In each case, the helix content is independent of concentration from 10 to 400 μM (data not shown). A similar alanine peptide with 13 contiguous alanines flanked by pairs of ornithine residues at each end was shown to be monomeric in an analytical ultracentrifugation study [80]. Thus, the helical structure in each peptide is stabilized by intramolecular interactions under the conditions studied.

The CD data suggest that the helical structure of EFE4-4 is significantly more stable than the control EFE5-5. Table 2 summarizes the CD data and free energy from the C-H···O H-bonding interaction. In the EFE4-4 peptide, two potential interactions with orientations of both Glu→Phe (N→C) and Phe→Glu (N→C) between the Glu and Phe side chains can contribute to the stability of the helix. However, the total free energy from these interactions is only −0.5 kcal mol^{-1}, determined by fitting the CD data to helix–coil transi-

Table 2
Helicity and energetics observed for the peptides

Peptide	$-[\theta]_{222}\times10^{-3}$ (° cm^2 dmol^{-1})[a]	f_H[b]	$\Delta\Delta G$ (kcal mol^{-1})[c]
EFE4-4	18.0	0.529	0.5
EFE5-5	15.3	0.450	–

[a] In 10 mM phosphate buffer pH 7 at 4°C. The peptide concentrations are 50 μM as determined by tyrosine absorbance at 275 nm.

[b] The relationship between fraction helicity and molar ellipticity is $f_H=[\theta]_{222}/[\theta]_{222}^{\circ}$, where $[\theta]_{222}^{\circ}=-34\,000$ is the estimated molar residue CD signal at 222 nm for an α-helix of 24 residues.

[c] The free energy of side chain interactions was computed using an algorithm based on the Zimm–Bragg helix–coil transition model, with the nucleation parameter $\sigma=0.004$ and values of the helix propensities from amino acids Ala, Glu, Phe, Tyr and Orn.

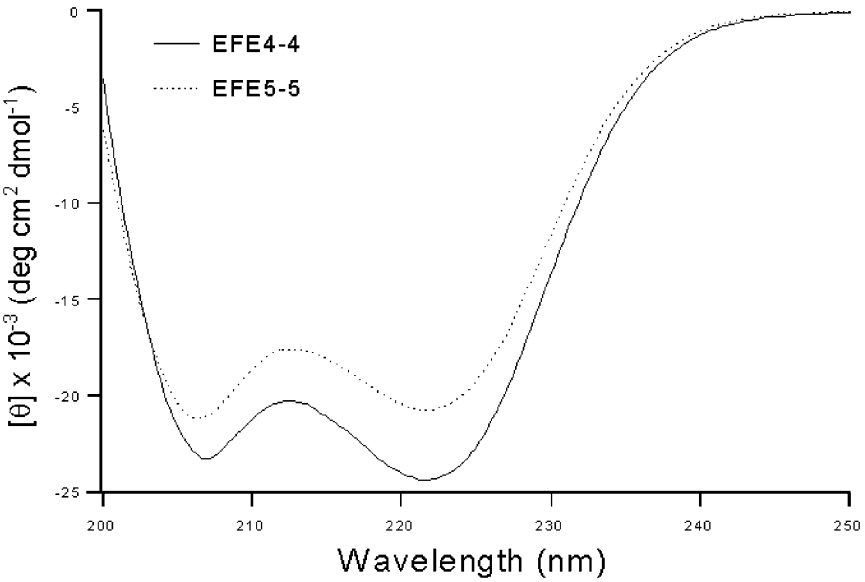

Fig. 1. Circular dichroism spectra of EFE4-4 and EFE5-5 in 10 mM phosphate buffer, pH 7, at 4 °C. The peptide concentrations are 50 μM in each case, as determined by tyrosine absorbance at 275 nm.

tion model. In EFR peptides [20,81], we detected an interaction between Glu and Phe side chains with the orientation Glu→Phe (N→C), and derived a free energy contribution of −0.53 kcal mol^{-1} by comparing the CD data of EFR4-4 to that of EFR5-4 and EFR4-5 to EFR5-5. Given that there is a negligible free-energy contribution from the potential cation−π interaction between Phe and Arg in EFR peptides, observed by comparing the CD data of EFR4-4 to that of EFR4-5 or EFR5-4 to EFR5-5, the derived free energy difference of −0.5 kcal mol^{-1} in the EFE4-4 relative to EFE5-5 must come from the Glu→Phe (N→C) interaction. Combining the results of the two studies, we can assign the free energy contribution of each Glu→Phe (N→C) interaction as −0.5 kcal mol^{-1}.

3.3. NMR analysis

NMR provides important structural details on side-chain–side-chain interactions in peptides. Since there is only one Phe residue in each peptide; we can directly assign the proton signals for Phe. There are two Glu residues in each peptide; however, there is only one Glu residue each in the EFR peptides studied previously [20]. The only difference in sequence between EFE5-5/EFE4-4 and EFR5-5/EFR4-4 is that position 17/16 is either Glu in EFE5-5/EFE4-4 or Arg in EFR5-5/EFR4-4. The residue in position 16 or 17 is too far away to interact with the Glu residue in position 8 or 7; hence the environment of the N-terminal Glu residues is similar between EFE4-4/EFE5-5 and EFR4-4/EFR5-5, and the chemical shifts of these Glu residues are in fact close to each other. The assignments of E8, F12 and E16 for EFE4-4 are shown in Fig. 2a; those for E7, F12 and E17 for EFE5-5 are shown in Fig. 2c. Strong nuclear Overhauser effect (NOE) interactions are detected between the side chains of F12 and E16, while the NOEs between E8 and F12 are weaker. As expected, we find no NOEs to F12 from either Glu side chain in EFE5-5.

As shown by CD data, the stabilization of EFE4-4 relative to EFE5-5 is mainly from the Glu→Phe (N→C) interaction. The NOE spectrum of EFE4-4 shows more contacts to Phe aromatic protons

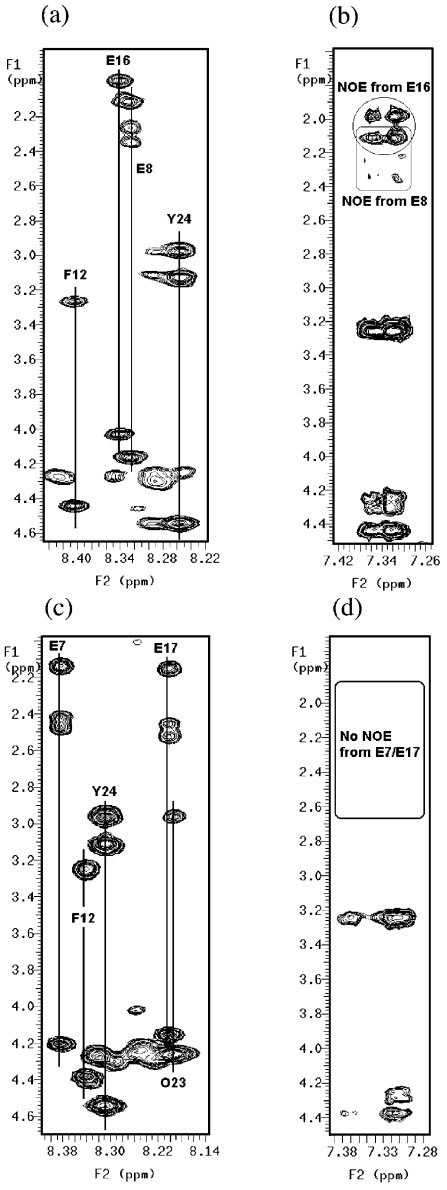

Fig. 2. Regions of TOCSY and NOESY spectra for EFE4-4 and EFE5-5 peptides. The strips of TOCSY spectra show the assignment of Glu and Phe side chains of the peptides. The NOESY spectra shown are the expanded regions where the possible NOE cross-peaks between Glu side chain and the Phe aromatic ring protons could be identified.

from the C-terminal Glu than from the N-terminal Glu. This inconsistency can be rationalized as follows: the interaction studied is that between a

CH from the edge of the aromatic ring and the O from the carboxyl group of Glu side chain. The distance between H and O in a C–H···O bond is approximately 2.7 Å. Both β and γ protons of Glu are at least three bonds away from the O atom, and hence the distance between aromatic CH and β/γ CH_2 of H-bonded Glu must be at least 4–4.5 Å and it is not surprising that we detect no or very weak NOEs between C–H···O bonded side chains. The NOEs detected between F12 and E16 cannot serve as evidence for the existence of C–H···O interaction between the two groups. This differs from the case in our study of cation–π interactions between Trp/Phe and Arg [81]. For cation–π interactions, the interactions are between the guanidine group of Arg and the centroid of the aromatic ring; NOEs between the side chains of Trp/Phe and Arg should be detected if cation–π interactions occur.

For both peptides, the NMR spectra show diagnostic features of typical helical peptides. In the NOESY spectrum (data not shown) of both peptides, strong $i-i+1$ amide–amide NOEs are detected across the whole backbone chain of the peptide, with some weak–medium-strength NOEs between i and $i+3/i+4$. Consistent with the CD spectra, this points to a large population of each peptide having α-helical conformation.

3.4. pH and salt titrations

As shown in Fig. 3a, changing pH from 7.0 to 3.0 makes EFE5-5 significantly more helical because the neutral form of Glu has a higher helix-forming propensity than the charged form [72,73]. The increase in helical content in EFE4-4 is significantly smaller than that in EFE5-5. This is consistent with the presence of C–H···O interactions in EFE4-4, but not in EFE5-5. Titrating the carboxyl group between pH 7.0 and 3.0 weakens the ability of O to serve as an H-bond acceptor, and thus cancels part of the effect from the increase in helix-forming propensity. Neutral salts were added to the peptide solutions to assess the dependence of C–H···O interactions on ionic strength. Both peptides show a similar trend in CD signals as a function of salt concentration (Fig. 3b) [13,82]: chloride salts stabilize helix up to about

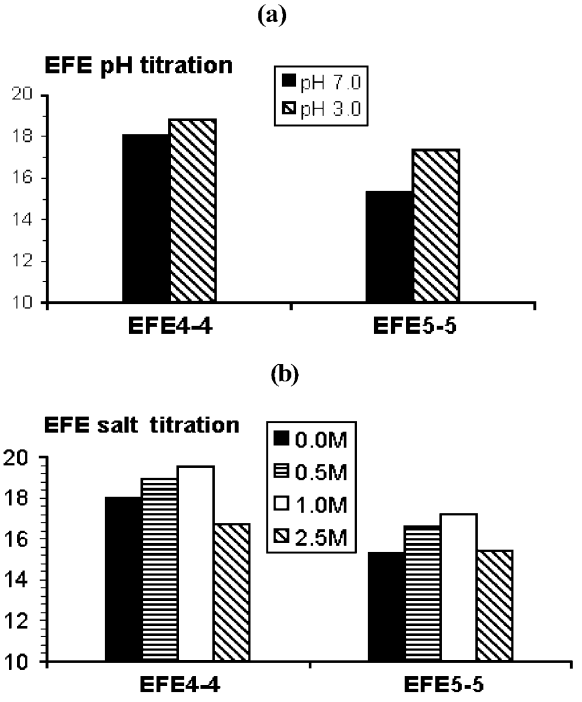

Fig. 3. (a) pH effect on the observed helicity of EFE4-4 and EFE5-5. (b) Salt concentration dependence of the observed helicity for EFE4-4 and EFE5-5. Comparison of the effect of increasing NaCl concentration on the C–H···O H-bonding interaction in model peptides.

1 M and then become destabilizing due to an apparent chaotropic effect. The data show no screening effect of salt on the C–H···O interaction. This result differs from what is observed for salt bridges, in which neutral salts screen the interaction, and peptides with salt bridges lose helicity relative to (or gain less helicity than) the control peptides as neutral salt is added [13,82].

4. Discussion

C–H···O H-bonds were originally discerned in high-resolution X-ray and neutron diffraction studies of small organic molecules [38]. Crystals of these molecules exhibit close C–H···O contacts and show the stereochemical hallmarks of hydrogen bonds. The idea was not confirmed until quite recently in proteins [26,27], in part because of the limitation in resolution. Many protein structures

determined by X-ray crystallography were analyzed using diffraction data that at best extend to a resolution of 1.5 Å, sufficient to obtain reasonably accurate coordinates for non-hydrogen atoms. Coordinates of hydrogen atoms can only be calculated from the coordinates of heavy atoms to which they are connected. The crystallographic refinement programs used unfortunately treat all close C···O interactions as repulsive [27].

Surveys of the Protein Data Bank (PDB) provide a wealth of information about geometries and stereochemistry of C–H···O H-bonds [27,28,39–42]. Despite observing numerous C–H···O contacts in proteins, major questions remain concerning their strength. A number of theoretical groups turned to *ab initio* quantum mechanical calculations to assess the energies of various interactions [62–66]. Thus, energies for the interactions $CH_4\cdots H_2O$, $C_2H_4\cdots H_2O$ and $C_2H_2\cdots H_2O$ have been estimated to be -0.49, -1.01 and -2.64 kcal mol^{-1}, respectively [83]. For the methane–water pair, the interaction energy increases systematically by approximately -1 kcal mol^{-1} as each hydrogen atom of methane is replaced by an electronegative atom, such as fluorine or chlorine, making CH a progressively stronger proton donor [62,64]. A recent calculation on the strength of the Cα–H···O H-bonds from amino acid residues showed that the binding energy of a Cα–H group to a water molecule lies in the range between -1.9 and -2.5 kcal mol^{-1} for non-polar and polar amino acids [66], approximately half of the binding energy of water to itself.

Although important information can be gained from these calculations, they estimate the energy for the interaction of a CH group with an O atom, and can only provide half the picture of the energy of C–H···O H-bonds. The net energy is the result of the energetic gain from the interaction studied and penalties, such as that of desolvation. Experimental results from double-cycle mutation analysis of proteins usually reflect a net balance between two counteracting components. However, there are numerous, weak non-covalent interactions in a protein molecule, and the contribution (especially net free energy contribution) from each individual interaction is small and hard to evaluate without confounding interference from proximal interactions. Thus, experimental analysis of any type of C–H···O H-bond may be extremely difficult. As discussed in Section 1, in model alanine-based α-helical peptides the background is simpler and can be specified more precisely. We take advantage of this model system here to study the net energetic contribution from putative C–H···O H-bonds between Phe and Glu side chains on the surface of a helix. We conclude that each surface C–H···O H-bond between Glu and Phe (N→C) can contribute approximately -0.5 kcal mol^{-1} to the stability of helical structure.

With regard to the obvious directionality effect shown by our data, similar effects have been observed in cation–π, and salt-bridge–side-chain interaction studies [12,13,84], and thus are not unexpected in our present study. The reason for such effects can be attributed to the existence of preferred rotamer patterns, depending on the side chain and the relative location of the partner side chains. In general, the more favorable interaction occurs when the longer side chain is located towards the C-terminal side ([13] and references therein).

Our result suggests that C–H···O H-bonds can in fact contribute significantly to protein/peptide stability, although the net effect may be less than classical N–H···O=C H-bonds. Theoretical calculations suggest that the interaction energy in a N–H···O=C H-bond is approximately -6 kcal mol^{-1} [85], whereas that for a C–H···O H-bond is between -1.9 and -2.5 kcal mol^{-1} [66], more than -3 kcal mol^{-1} less than the former. However, the net value of -0.5 kcal mol^{-1} for C–H···O H-bonds is comparable to that of a N–H···O=C H-bond, given the free energy of the latter is approximately -0.4 kcal mol^{-1} [86]. One possible explanation is that the desolvation cost for C–H···O H-bonds is also approximately 3 kcal mol^{-1} less than that for N–H···O=C H-bonds.

What can we learn from the presence of C–H···O H-bonds in stabilizing helical structure? McDonald and Thornton [87] suggested that each backbone amide in a protein tends to satisfy its individual potential to form H-bonds. There is only one NH and C=O in the backbone of each residue; each NH group can usually serve as one H-donor, while each C=O has two lone electron pairs with

potential to form two H-bonds. This means that NH as a group can satisfy its potential to form H-bonds, whereas approximately half of potential of the C=O group may be unsatisfied if we consider only classical H-bonds. Baldwin has pointed out that one consequence of this situation is a preferential hydration of C=O groups in isolated α-helices [88]. Apparently any C_α–H donor, aromatic side-chain CH group or other CH groups adjacent to electronegative groups may be able to satisfy the C=O H-bonding potential, as has been suggested in β-sheets [27], the collagen triple helix [39] and α-helices containing proline residues [40].

As we have mentioned, the understanding and even confirmation of the existence of C–H···O H-bonds have been hindered by the crystallographic refinement programs used, which treat all close C···O interactions as repulsive. Derewenda et al. [27] urged force field developers to reconsider this feature in the programs being used after they found that a large percentage of short C···O contacts appear to constitute cohesive interactions. Still they could not provide theorists with any substantial quantitative energetic information. Thus, our present results could provide fundamental information on C–H···O H-bonds for attempts to refine existing force-field parameters.

The program AGADIR [21,22] is now extensively used to estimate the helical content of any peptide from its primary sequence. In order to check if AGADIR takes C–H···O H-bonding interactions into account in calculating helical content, we used two sequences, EFE5-5_{test} and EFE4-4_{test}, based on peptides of this study. The sequences of EFE5-5_{test} and EFE4-4_{test} are AcAAAAEAAAA-FAAAAEAAAANH$_2$ and AcAAAAAEAAAF-AAAEAAAAANH$_2$, respectively. To our surprise, the calculated helix content of EFE5-5_{test} is 50.3%, significantly larger than that for EFE4-4_{test}—40.7%—under the same conditions: pH 7.00 at 278 K with an ionic strength of 0.01 M. Thus, it seems C–H···O H-bonding interactions are not taken into account in the interaction library of AGADIR.

The balance of forces that maintain the native state of α-helix is less simple than has been previously thought [2]. In part, this reflects a number of non-covalent interactions that influence the ability of side chains to interact, favorably or unfavorably. Our present study shows that C–H···O H-bonds have a role in these interactions, and could make a significant contribution to the native state of proteins as well. A different complication in understanding helical stability stems from the nature of the unfolded states that represent structures alternative to α-helical or β-sheet conformations. Effects of this kind were predicted by Pappu et al. [89], who argue that steric effects alone significantly diminish the phase space accessible to a disordered polypeptide. In a study of unfolded oligo-(alanine), we find that the predominant structure is PPII, not β-sheet or a blend of different conformations expected from studies of random coils ([90,91]). This means that α-helix forms in water mostly at the expense of PPII helix, rather than beta strands or a mix of conformations. The implication of this for the interactions that stabilize α-helix [92,93] is that we now have to account for effects that can either stabilize the former directly or act via destabilizing PPII conformation.

As one referee pointed out, the stabilizing interaction between Glu and Phe that we attribute to a C–H···O H-bonding interaction could in principle originate from sources other than this specific effect [42,94]. Our data *per se* cannot allay this concern conclusively, since our spectra do not directly demonstrate the H-bonding interaction. On physical grounds, however, we prefer to interpret the interaction as a type of C–H···O H-bond. Most obvious alternatives can be excluded: for example, the negative charge on Glu eliminates charge–π types of interaction (as the centroid of the π-ring is negatively charged). We believe more experimental and theoretical data are required to resolve this important issue definitively.

5. Conclusion

We have used a combination of circular dichroism and nuclear magnetic resonance to investigate the helix content of two peptides in which Phe and Glu residues are located at $i,i+4$ or $i,i+5$ positions. Analysis of the free energy contribution of the C–H···O interaction using a modified

Zimm–Bragg multi-state helix–coil transition model shows that a putative C–H···O interaction between Phe and Glu side chains contributes favorably to helix stability. Combined with our previous studies on EFR peptides [20], the results suggest that Glu/Phe C–H···O H-bonding interactions stabilize helical structure only in the orientation Glu→Phe $(i,i+4)$ (N→C). Each Glu→Phe $(i,i+4)$ (N→C) interaction can contribute approximately -0.5 kcal mol^{-1} to the stability of helical peptide. Estimates of the strength of the peptide N–H···O=C H-bonds are comparable to this number. The reverse orientation Phe→Glu $(i,i+4)$ (N→C) appears to contribute negligibly. pH titration provides further support for the existence of C–H···O H-bonds. The C–H···O H-bonding interactions studied here are insensitive to the screening effect of neutral salt.

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