Measuring the Strength of Side-Chain Hydrogen Bonds in Peptide Helices: The Gln•Asp (i, i + 4) Interaction†

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ABSTRACT: Whether hydrogen bonds between side chains are energetically significant in proteins and peptides has been controversial. A method is given here for measuring these interactions in peptide helices by comparing the helix contents of peptides with 1, 2, or 3 interactions. Results are given for the glutamine-aspartate (i, i + 4) hydrogen-bond interaction. The Gibbs energy of the interaction is -1.0 kcal/mol when aspartate is charged and -0.4 kcal/mol when it is protonated. Magnetic resonance experiments show that the aspartate carboxylate group interacts specifically with the trans amide proton (H₂E) of glutamine. The interaction is observed only when the glutamine residue is N-terminal to the aspartate and when the spacing is (i, i + 4). The same stereochemistry is found in protein structures, where the (i, i + 4) glutamine-aspartate interaction occurs much more frequently than other possible arrangements.

Hydrogen-bond (H-bond) interactions between side chains are found frequently in protein structures. In a study of 42 proteins (Stickle et al., 1992), an average of 18 side-chain H-bonds per 160 residues was found. Thus, if each side-chain H-bond contributes -1 kcal/mol to ΔG° for folding, the overall contribution would be -18 kcal/mol for a 160-residue protein. This free energy would tip the balance between stable and unstable folding, given that values of ΔG° for typical protein-folding reactions are between -5 and -15 kcal/mol (Pace, 1990). Information about the energetics of side-chain H-bond interactions is quite limited and comes chiefly from directed mutagenesis experiments on proteins, including T4 lysozyme (Alber et al., 1987), barnase (Chen et al., 1993), ribonuclease T1 (Shirley et al., 1992), and staphylococcal nuclease (Thorson et al., 1995): see below.

Analyzing side-chain interactions in peptide helices has several desirable properties. Fairly weak interactions produce significant changes in the fraction helix. Both natural and non-natural amino acids can be studied. The necessary peptides can be made readily. The helix-coil transition theory of Lifson and Roig (1961) is well-suited to analyze helix formation by peptides with known sequences (Chakrabarty et al., 1991), and it is readily extended to include side-chain interactions (Scholtz et al., 1993). The theory has been modified to include N-cap and C-cap interactions (Doig et al., 1994). The parameters needed to use the theory, including the helix nucleation parameter (Scholtz et al., 1991a; Rohl et al., 1992), the helix propensities in alanine-based peptides (Chakrabarty et al., 1994), and the N-cap and C-cap propensities (Doig & Baldwin, 1995), have all been determined.

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1 Abbreviations. CD, circular dichroism; NMR, nuclear magnetic resonance; Gln, glutamine; Asp, aspartate; Glu, glutamate; HOBT, 1-hydroxybenzotriazole; TSP, 3-(trimethylsilyl)propionic acid; PDB, protein data bank; Thr, threonine; Asn, asparagine.
The H-bond interaction studied here, Gln-Asp \((i, i + 4)\), was first observed unexpectedly in a study of how the interaction between charged aspartate and the helix dipole depends on the aspartate position (Huyghues-Despointes et al., 1993a), and a parallel observation of a Gln-Glu \((i, i + 4)\) interaction (Scholtz et al., 1993) was also made. We set out to find methods of detecting and measuring the interaction that would leave no doubt about its reality. We describe here one method of detecting the Gln-Asp interaction, which also gives information about its stereochemistry, and a separate method of measuring the strength of the interaction.

**EXPERIMENTAL PROCEDURES**

**Peptide Synthesis and Purification.** Peptides were synthesized by the solid-phase method using a 5:5-coupling procedure, employing pentafluorophenyl esters of then coupled to the growing peptide chain by using BOP (Millipore). The Fmoc-amino acid derivative of L-Gln synthesized by the solid-phase method using an active ester-titration that would leave no doubt about its reality. We describe Isotope Laboratory) was made (Stewart et al., 1984) and also gives information about its stereochemistry, and a separate method of measuring the strength of the interaction.

**Circular Dichroism Spectroscopy.** CD was measured with an AVIV 60 DS spectropolarimeter. The ellipticity was calibrated with (-)-10-camphorsulfonic acid. Cuvettes with either a 1-mm or a 1-cm path length were used. Samples for CD measurements were prepared as described by Huyghues-Despointes et al. (1993). The peptide purity was assessed by C18 reverse-phase chromatography using the Pharmacia FPLC system; purity was greater than 95% for each peptide. The primary- ion molecular weight of each peptide was confirmed by fast atom bombardment or electrospray mass spectrometry. The conditions for storing and handling the peptides were described elsewhere (Huyghues-Despointes et al., 1993a).

**NMR Spectroscopy.** All NMR spectra were recorded at 2 °C on a 500-MHz General Electric GN-Omega spectrometer, using a 6000-Hz spectral width for one-dimensional data collection. Spectra were processed on Silicon Graphics Personal Iris and Indigo computers using FELIX (Hare Research, Inc.). Samples for pH titration were prepared by making a 1-2 mM peptide solution containing 10 mM NaCl, 7% D2O, and 3 mM sodium phosphate. The free induction decay was the sum of 16–64 scans consisting of 4096 complex points. The pH dependence of chemical shifts was measured against the TSP standard, after taking account of the pH dependence of chemical shift of TSP (De Marco, 1977). The assignments of the glutamine cis and trans amide protons are based on the reported chemical shifts of several primary amide compounds (Redfield & Waelder, 1979).

**Data Analysis.** The standard Gibbs energy, \(\Delta G^\circ\), of the interaction was computed from the fraction helix by an algorithm (Stapley et al., 1995) that incorporates the interaction into the Lifson and Roig theory (1961) [see also Scholtz et al. (1993)]. The “homopolymer approximation” was used: the reference peptide was treated as a homopolymer, except for the Asp residues and an equal number of corresponding Gln residues, and an average helix propensity \(p\) was computed. This value of \(\langle p\rangle\) was assigned to all residues in the test peptide except for the Asp residues and the interacting Gln residues. The values used for the helix propensities (Chakrabarty et al., 1994) and nucleation parameter (Rohl et al., 1992) were \(w_0 = 0.56\), \(w_D = 0.35\), and \(v = 0.048\). The interaction is included by assigning an additional statistical weight \(p\) to conformations in which residues \(i\) through \(i + 4\) are helical, as defined by the backbone \(\phi\) and \(\psi\) angles. The interaction parameter (see Table 1) is defined as the equilibrium constant for the interaction in a completely helical peptide. This equilibrium constant is related to the standard Gibbs energy by the relation \(\Delta G^\circ = -RT \ln p\). In peptides with two or more interacting residue pairs, the interactions are treated as being independent of each other.

**Protein Structure Searches.** The protein sequences were extracted from a nonhomologous set of chains from the Brookhaven Protein Data Bank (Berstein et al., 1977). A detailed description of the procedures is given by Klinger and Bruttig (1994). The set includes 212 high-resolution (>2.5 Å), nonhomologous, nonmutant, nonmodel structures. We use the Iditis program from Oxford Molecular (Thornton & Gardner, 1989), a program for querying the PDB in relational form to extract sequences of specific secondary structure assigned by the extended DSSP method (Kabsch & Sander, 1983) and implemented in Iditis. The helices are identified by hydrogen bonding, and the program includes one residue at each end of the helix, usually at the capping positions. The expected number of \((i, i + 4)\) XY pairs is

**Table 1. The Gibbs Energy of the Gln-Asp \((i, i + 4)\) H-Bond in Helical Peptides**

<table>
<thead>
<tr>
<th>pH</th>
<th>Peptide</th>
<th>(f_H)</th>
<th>(f_H^{\text{ref}})</th>
<th>(p)</th>
<th>(\Delta G^\circ) (cal/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH 2</td>
<td>1QD</td>
<td>0.35</td>
<td>0.26</td>
<td>2.7</td>
<td>-540</td>
</tr>
<tr>
<td></td>
<td>2QD</td>
<td>0.42</td>
<td>0.20</td>
<td>2.1</td>
<td>-390</td>
</tr>
<tr>
<td></td>
<td>3QD</td>
<td>0.53</td>
<td>0.26</td>
<td>1.9</td>
<td>-350</td>
</tr>
<tr>
<td>pH 7</td>
<td>1QD</td>
<td>0.48</td>
<td>0.29</td>
<td>5.0</td>
<td>-870</td>
</tr>
<tr>
<td></td>
<td>2QD</td>
<td>0.61</td>
<td>0.11</td>
<td>7.1</td>
<td>-1060</td>
</tr>
<tr>
<td></td>
<td>3QD</td>
<td>0.75</td>
<td>0.15</td>
<td>6.2</td>
<td>-990</td>
</tr>
</tbody>
</table>

*Conditions: 10 mM NaCl, 0 °C; similar results were obtained in 1 M NaCl. The peptides contain either 1, 2, or 3 interacting pairs of Gln. Asp residues, as indicated: see Figure 2a for their sequences and those of the reference peptides. The apparent equilibrium constant for the interaction: \(\Delta G^\circ = -RT \ln p\). The standard Gibbs energy of the Gln-Asp \((i, i + 4)\) interaction, given per interaction.
FIGURE 1: Detection of the Gln-Asp (i, i + 4) interaction and identification of the interacting Gln amide proton by pH titration of chemical shifts. A. 1H-NMR spectrum of peptide Q3D7 in 10 mM NaCl pH 7.4, 2 °C. The amide group of 43 is labeled with 15N; the wide splitting of each 15NH resonance is used to identify the amide protons of Q3. H2 is the trans proton, and H1 is the cis amide proton (see inset). The sequence of Q3D7 is Ac-AAQ-AAAQAAADDQAAAAY-NH2. B. Titration curves of the H2 (●) and H1 (○) resonances of Q3 and of the β-CH2 (×) resonance of D7.

calculated from the product of the frequency of residue X at position i and the frequency of residue Y at position i + 4; the expected number of (i, i + 4) YX pairs is calculated from the product of the frequency of residue Y at position i and the frequency of residue X at position i + 4. Since these frequencies may be different, the expected number of XY (i, i + 4) pairs may not be similar to the expected number of YX (i, i + 4) pairs. In the distance-based identification of H-bonds in the structure set, the maximum allowed distance between the donor and acceptor atoms is 3.9 Å.

RESULTS

pH Titration of Chemical Shifts. To identify the two interacting groups, the amide nitrogen of the glutamine residue in the Gln-Asp (i, i + 4) residue pair was labeled with 15N (see Experimental Procedures). The label causes a characteristic splitting of each amide proton resonance, shown in Figure 1A. Generally, the H2 (trans) proton is found downfield of the H1 (cis) proton in primary amides (Redfield & Waelder, 1979). Measurement of the pH titration curves of these resonances (Figure 1B) shows that only the H2 proton changes its chemical shift as the aspartate carboxylate group titrates. There is a substantial change between pH 6 and pH 2 in the chemical shift of the trans resonance (0.22 ppm) but no measurable change in the cis resonance. No change in the chemical shift of any Gln amide

resonances is found in peptides with either the Asp-Gln (i, i + 4) or the Gln-Asp (i, i + 3) sequences (data not shown). Thus, the amide group of Gln i interacts specifically with the aspartate residues are the same, or nearly the same, compositions of both peptides are the same, and the positions of the aspartate residues are the same, or nearly the same, as in the test peptide. These are the main variables, other than specific side-chain interactions and electrostatic interactions, that affect the helix content. The position of a charged aspartate residue affects the helix content via both its low helix propensity and the charge–helix dipole interaction (Huyghues-Despointes et al., 1993a). These effects can be seen in the reference peptides. The fact that the helix contents of the reference peptides do not change in a regular manner as the number of aspartate residues is varied emphasizes that the length of the peptide and the position of the charged aspartate residues are also variables that

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>1QD</td>
<td>Ac-AAQAADAQAAAQAAY-NH2</td>
</tr>
<tr>
<td>1QD ref</td>
<td>Ac-AQAADDQQAAAQAAY-NH2</td>
</tr>
<tr>
<td>2QD</td>
<td>Ac-AAQAADAQAAADDQAAY-NH2</td>
</tr>
<tr>
<td>2QD ref</td>
<td>Ac-AQAADAQAAAADAAAAGY-NH2</td>
</tr>
<tr>
<td>3QD</td>
<td>Ac-AAQAADAQAAAADDQAADAAAAGY-NH2</td>
</tr>
<tr>
<td>3QD ref</td>
<td>Ac-AQAADAQAAAADAQAAAADAAAAGY-NH2</td>
</tr>
</tbody>
</table>

FIGURE 2: Measurement by circular dichroism of the increase in helix content caused by the Gln-Asp (i, i + 4) interaction in peptides with 1, 2, or 3 interacting pairs of Gln, Asp residues. A. Sequences of the peptides studied and of the corresponding reference peptides. B. pH titration curves of helix content measured by circular dichroism. Filled symbols show the test peptides, open symbols show the corresponding reference peptides: (●) 1QD, (▲) 2QD, (■) 3QD. [Φ]222 is the mean residue ellipticity measured at 222 nm.

Helix Contents of Peptides with 1, 2, and 3 Gln-Asp (i, i + 4) Pairs. To measure the standard Gibbs energy of the Gln-Asp (i, i + 4) interaction, peptides were made that contain either 1, 2, or 3 Gln-Asp (i, i + 4) pairs and their helix contents were measured by circular dichroism. The change in helix content with pH was also measured to determine the effect of protonating the aspartate residues. Matching reference peptides were made, and their helix contents and pH titration curves were measured. The peptide sequences are given in Figure 2A, and the titration curves of helix content are given in Figure 2B.

It is essential to design a reference peptide that matches as closely as possible the test peptide and lacks the Gln-Asp (i, i + 4) interaction. In each reference peptide used here, the length is the same as in the test peptide, the amino acid compositions of both peptides are the same, and the positions of the aspartate residues are the same, or nearly the same, as in the test peptide. These are the main variables, other than specific side-chain interactions and electrostatic interactions, that affect the helix content. The position of a charged aspartate residue affects the helix content via both its low helix propensity and the charge–helix dipole interaction (Huyghues-Despointes et al., 1993a). These effects can be seen in the reference peptides. The fact that the helix contents of the reference peptides do not change in a regular manner as the number of aspartate residues is varied emphasizes that the length of the peptide and the position of the charged aspartate residues are also variables that

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significantly affect the helix content. An increase in helix content with decreasing pH is observed for 2QDref and 3QDref. An unfavorable interaction between aspartate near the C-terminus and the helix dipole is removed upon protonation of aspartate. This effect is not observed for 1QDref because the single aspartate is near the center of the peptide. The glutamine residues in all three reference peptides are spaced either (i, i + 2) or (i, i + 3) from the nearest aspartate. Previous data show that glutamine and aspartate do not interact in these spacings (Huyghues-Despointes et al., 1993a).

Figure 2 shows that the helix content of each test peptide is greater than that of its reference peptide, both at pH 2 and at pH 7, that the difference in helix content between the test and reference peptide increases with the number of Gln*Asp pairs, and that the difference in helix content follows the pH titration curve of aspartate, and is greater at pH 7 than at pH 2. The helix content does not scale linearly with the interaction strength (just as an equilibrium constant does not scale linearly with the amount of product in a reaction), and there is a larger difference in helix content between 1QD and 2QD than between 2QD and 3QD, at both pH 2 and pH 7. These results show that the Gln*Asp (i, i + 4) interaction is effective in stabilizing peptide helices and that the interaction persists at pH 2, where aspartate is protonated, although the interaction is stronger at pH 7 than at pH 2.

Table 1 shows that, within error, the value for the $\Delta G^o$ of the interaction is the same, per interaction, in peptides with 1, 2, or 3 interactions. At pH 2, where an uncharged Gln*Asp H-bond is made, $\Delta G^o = -0.4$, kcal/mol, while at pH 7, where a charged H-bond is made, $\Delta G^o = -1.0$, kcal/mol.$^{-1}$. The measurements of helix content and of $\Delta G^o$ were repeated in 1 M NaCl to find out if screening of electrostatic interactions between aspartate residues is important. Similar values of $\Delta G^o$ were obtained in 1 M NaCl as at 10 mM NaCl, both at pH 7 and pH 2 (data not shown).

### Table 2. Frequencies of (i, i + 4) Side-Chain H-Bond Interactions in Protein Helices

<table>
<thead>
<tr>
<th>sequence</th>
<th>no.</th>
<th>H-bond$^a$</th>
<th>$i$ trans$^b$</th>
<th>$i + 4$ gauche$^c$</th>
</tr>
</thead>
<tbody>
<tr>
<td>QE</td>
<td>52</td>
<td>18</td>
<td>32 (21.4)</td>
<td>25 (31.8)</td>
</tr>
<tr>
<td>EQ</td>
<td>29</td>
<td>2</td>
<td>11 (11.4)</td>
<td>18 (18.0)</td>
</tr>
<tr>
<td>DQ</td>
<td>22</td>
<td>1</td>
<td>5 (3.9)</td>
<td>13 (13.6)</td>
</tr>
</tbody>
</table>

$^a$ The number of sequences with (i, i + 4) spacing in protein helices; the expected number, based on single-residue frequencies, is given in parentheses. $^b$ The average Gln*Asp H-bond distance is 3.1 ± 0.2 A; the average Glu*Glu H-bond distance is 3.1 ± 0.5 A. None of the Glu*Asp H-bond interactions is at the N- or C-cap position. $^c$ The number of instances that residue i has a trans $\chi_i$ rotamer; the expected number is given in parentheses. $^d$ The number of instances that residue i + 4 has a gauche$^+\ +\ \chi_i$ rotamer; the expected number is given in parentheses.

These results show that the Gln*Asp (i, i + 4) interaction occurs commonly in protein helices. The charged H-bond interaction at pH 7 is stronger than the H-bond made between glutamine and protonated aspartate at pH 2, but the uncharged H-bond is also helix-stabilizing. The value of $\Delta G^o$ found here for a side-chain H-bond is substantially smaller than the values reported from mutagenesis experiments in proteins (Fersht et al., 1985; Alber et al., 1987; Shirley et al., 1992; Chen et al., 1993; Thorson et al., 1995). At least two main factors are involved. First, side chains are more constrained in proteins than in alanine-based peptide helices, so there is a greater loss of side-chain conformational entropy when the interaction is formed in a peptide helix. Second, a polar side chain that is buried in a protein without another H-bonding group nearby is often unable to H-bond to water, whereas in a peptide helix it would be able to H-bond to water. Consequently, the penalty for breaking the H-bond by directed mutagenesis is larger in the protein than in the peptide. Because structural rearrangements are likely to occur in proteins, it is important to determine the X-ray structures of the mutants. Data for mutants in which the H-bond is broken and no major structural rearrangement occurs were reported for three cases (Leu, Ala, Phe) in T4 lysozyme, with $\Delta\Delta G^o$ values of 1.3, 1.4, and 2.4 kcal/mol (Alber et al., 1987), respectively, and for two mutants of barnase with $\Delta\Delta G^o$ values of 1.4 and 1.9 kcal/mol (Chen et al., 1993), respectively. A new approach to the problem has just been reported (Thorson et al., 1995) in which a non-natural amino acid can be introduced at a specific location via protein synthesis in vitro, so that the H-bond can be left intact but the strength of the H-bond can be varied over wide limits. Values of $-1.5$ to $-2.0$ kcal/mol were found for the Tyr-Glu H-bond interaction at two sites in staphylococcal nuclease. An NMR study of the effects of specific side-chain interactions in a histidine-containing protein has just appeared (Hammen et al., 1995). The results reported here add to the evidence that H-bonds are important in stabilizing $\alpha$-helices in water. The favorable

**Discussion**

**$H$-Bonds in Peptide Helices Compared to Proteins.** These results show clearly that the Gln*Asp (i, i + 4) H-bond interaction stabilizes peptide helices and that the same interaction occurs commonly in protein helices. The charged H-bond interaction at pH 7 is stronger than the H-bond made between glutamine and protonated aspartate at pH 2, but the uncharged H-bond is also helix-stabilizing. The value of $\Delta G^o$ found here for a side-chain H-bond is substantially smaller than the values reported from mutagenesis experiments in proteins (Fersht et al., 1985; Alber et al., 1987; Shirley et al., 1992; Chen et al., 1993; Thorson et al., 1995). At least two main factors are involved. First, side chains are more constrained in proteins than in alanine-based peptide helices, so there is a greater loss of side-chain conformational entropy when the interaction is formed in a peptide helix. Second, a polar side chain that is buried in a protein without another H-bonding group nearby is often unable to H-bond to water, whereas in a peptide helix it would be able to H-bond to water. Consequently, the penalty for breaking the H-bond by directed mutagenesis is larger in the protein than in the peptide. Because structural rearrangements are likely to occur in proteins, it is important to determine the X-ray structures of the mutants. Data for mutants in which the H-bond is broken and no major structural rearrangement occurs were reported for three cases (Leu, Ala, Phe) in T4 lysozyme, with $\Delta\Delta G^o$ values of 1.3, 1.4, and 2.4 kcal/mol (Alber et al., 1987), respectively, and for two mutants of barnase with $\Delta\Delta G^o$ values of 1.4 and 1.9 kcal/mol (Chen et al., 1993), respectively. A new approach to the problem has just been reported (Thorson et al., 1995) in which a non-natural amino acid can be introduced at a specific location via protein synthesis in vitro, so that the H-bond can be left intact but the strength of the H-bond can be varied over wide limits. Values of $-1.5$ to $-2.0$ kcal/mol were found for the Tyr-Glu H-bond interaction at two sites in staphylococcal nuclease. An NMR study of the effects of specific side-chain interactions in a histidine-containing protein has just appeared (Hammen et al., 1995). The results reported here add to the evidence that H-bonds are important in stabilizing $\alpha$-helices in water. The favorable
enthalpy of forming the helix backbone (~1 kcal per mole of residues) is the major factor driving formation of alanine-based peptide helices in water (Scholtz et al., 1991a,b), and formation of the peptide H-bond is an important factor in determining this enthalpy change. Note, however, that this favorable enthalpy of stabilizing an isolated helix may change when the helix is buried in a protein and the backbone is desolvated (Ben-Naim, 1991; Yang et al., 1992). The N-cap interaction made between a polar side chain such as Asn or Thr at N-cap and an unsatisfied main-chain NH group was first detected in protein helices (Presta & Rose, 1988; Richardson & Richardson, 1988) and was later studied in peptide helices, where there is now general agreement that this side-chain–main-chain H-bond makes an important contribution to helix stability (Bruch et al., 1991; Forood et al., 1993; Lyu et al., 1993; Chakrabarty et al., 1993b). Side-chain–side-chain H-bonds can now be added to this list.

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