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Effect of the Substitution Ala → Gly at Each of Five Residue Positions in the C-Peptide Helix†

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ABSTRACT: The substitution Ala → Gly has been studied in a unique-sequence peptide (related in sequence to the C-peptide of ribonuclease A) to determine its effect on C-peptide helicity at different residue positions. There is a substantial decrease in helicity for Ala → Gly at residue position 4, 5, or 6 but only a small decrease in helicity for Ala → Gly at end residue 1 and no decrease at end residue 13. The change for Ala → Gly is similar at position 4, 5, or 6; the change is caused chiefly by the difference in s, the helix growth parameter in the Zimm–Bragg model for α-helix formation, between Ala and Gly. Thus, the helicity of C-peptide depends sensitively on s at interior positions. The small change in helicity found for Ala → Gly at either end position suggests that the end residues are largely excluded from the helix, with the result that helicity is relatively unaffected by replacement of an end residue. Another possibility is that some helix-stabilizing effect is exerted by Gly only at an end position. Exclusion of an end residue from the helix might be caused either by fraying of the helix ends or by helix termination at an interior residue, resulting from a helix stop signal such as the Glu-2$^-$–Arg-10$^+$ salt bridge or the Phe-8–His-12$^+$ ring interaction.

The relative frequency (P_α) with which an amino acid occurs in protein α-helices can be correlated with s, the Zimm–Bragg helix growth parameter (Chou & Fasman, 1974). The correlation is, however, imperfect [see p 331 of Creighton (1984) and references cited therein]; Tyr has a considerably lower P_α value than would be expected on the basis of its value for s, whereas Gln, Ala, Leu, Lys$^+$, Asp$^-$, Glu$^-$, and Met all have higher P_α values than expected. It would be desirable to have an independent method of measuring the helix-stabilizing tendency of an amino acid, in addition to the host–guest method (Sueki et al., 1984), to investigate possible reasons for the deviations from an exact correlation.

The C-peptide of RNase A (a peptide containing the 13 N-terminal residues) provides a possible system for studying the deviations from an exact correlation. C-Peptide shows readily measured helicity in H_2O at 0 °C in a monomolecular reaction (Brown & Klee, 1971; Bierzynski et al., 1982), and derivatives of C-peptide have been found that are stronger helix formers (Shoemaker et al., 1987a). Since it has a unique sequence and contains several different amino acids, C-peptide provides a different type of helix-forming system than the random-sequence copolymers used in host–guest studies (Sueki et al., 1984). One aim of this paper is to test the suitability of C-peptide as a system in which to correlate the helix-stabilizing tendency of an amino acid with its frequency in α-helices in proteins. A second aim is to investigate the factors involved in a systematic study of position-dependent effects on helicity. The frequencies of the acidic and basic residues are quite different from each other at the N-terminus and, in an inverse manner, at the C-terminus of protein α-helices [Cook, 1967; see Chou and Fasman (1978) and references cited therein]. This effect may be explained by the helix-stabilizing interactions of these charged residues with the helix dipole (Ptitsyn & Finkelstein, 1970; Blagdon & Goodman, 1975; Shoemaker et al., 1987a), but some uncharged residues [e.g., Trp; Chou & Fasman, 1978] also show strikingly asymmetric patterns of frequency versus helix position, and

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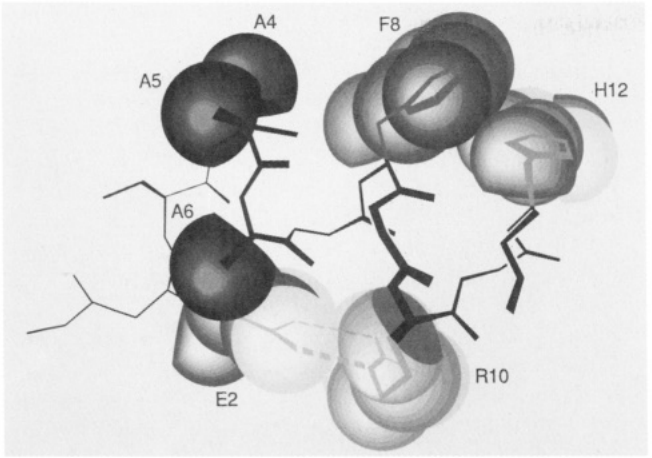

FIGURE 1: Computer graphics view of the C-peptide helix taken from the X-ray structure of RNase A (Wlodawer & Sjölin, 1983), showing the absence of contacts between the $-Ch_3$ side chains of Ala-4, -5, and -6, and residues which participate in side chain interactions that stabilize the helix (Glu-2^-–Arg-10^+, Phe-8–His-12^+).

the reasons for this behavior are not known.

Another reason for undertaking this study is to provide data needed in the analysis of the Glu-2^-–Arg-10^+ and Phe-8–His-12^+ side chain interactions in the C-peptide helix. This study of the Ala → Gly replacement is intended to serve as a model for study of Ala → Glu, Ala → Arg, Ala → Phe, and Ala → His replacements in later work. The position-independent effects of these replacements are needed to complete the analysis of the position-dependent interactions between Glu-2^- and Arg-10^+ and between Phe-8 and His-12^+.

We used the following two criteria in deciding if the C-peptide system provides a suitable base for investigating the correlation of P_α with helix-stabilizing tendency. (1) If the C-peptide system is to be experimentally useful, then a replacement such as Ala → Gly, which is accompanied by a substantial change in s, must produce an important change in helicity. (2) If the results are to be represented by a straightforward correlation of P_α with helix-stabilizing tendency, then similar changes in helicity should be found when a given substitution is made at different (interior) residue positions.

We chose as a test system the substitution Ala → Gly for the following reasons. (i) Ala occurs at three different interior positions (4, 5, and 6) in C-peptide, unlike any other residue. (ii) In the helix defined by the X-ray structure of RNase A (Wlodawer & Sjölin, 1983), the $-CH_3$ side chain of Ala at position 4, 5, or 6 (see Figure 1) does not contact either of the two interacting pairs of side chains, Glu-2^-–Arg-10^+ and Phe-8–His-12^+, that are important for the stability of the C-peptide helix. (iii) Gly has the lowest s value of any amino acid yet measured by the host–guest method (Sueki et al., 1984), and there is a large change in s for Ala → Gly. (iv) Since the side chain of Gly is smaller than that of Ala, Gly also should not contact the sidechains of Glu-2, Arg-10, Phe-8, or His-12 when Gly is at position 4, 5, or 6.

Instead of C-peptide itself, we use as a starting point the derivative peptide RN21 (reference peptide III; Shoemaker et al., 1987a) which is a stronger helix former. Since RN21 contains acetyl-Ala-1 and Ala-13-amide as end residues, the effect of the substitution Ala → Gly can also be studied at the two end residues.

Materials and Methods

The peptides were synthesized by the solid-phase method as described by Marqusee and Baldwin (1987). They were purified by reverse-phase chromatography using a C18 resin and a gradient of 10–50% acetonitrile in 0.1% trifluoroacetic acid. Peptide purity and amino acid compositions were checked by FPLC and amino acid analysis. The correct primary ion molecular weights were confirmed by FAB mass spectrometry.

Peptide concentrations of ~1 mM stock solutions in H_2O were found by using multiple determinations by the ninhydrin method (Rosen, 1957). The ninhydrin method was checked by using a peptide containing a single tyrosine residue as a standard. The concentration of the tyrosine-containing peptide was determined by the absorbance at 275 nm. The buffer used for CD measurements was 0.1 M NaCl, 1 mM sodium citrate, 1 mM sodium borate, and 1 mM sodium phosphate; the pH was adjusted with HCl and NaOH. The concentration used for CD measurements was around 15–20 μM.

CD measurements were made on a modified Cary 60 spectropolarimeter (Aviv Associates Model 60 DS), using cuvettes with a 10-mm path length. The instrument was calibrated with (+)-10-camphorsulfonic acid (Chen & Yang, 1977). Unless otherwise noted, the mean residue ellipticity at 222 nm, $[\theta]_{222}$, is reported.

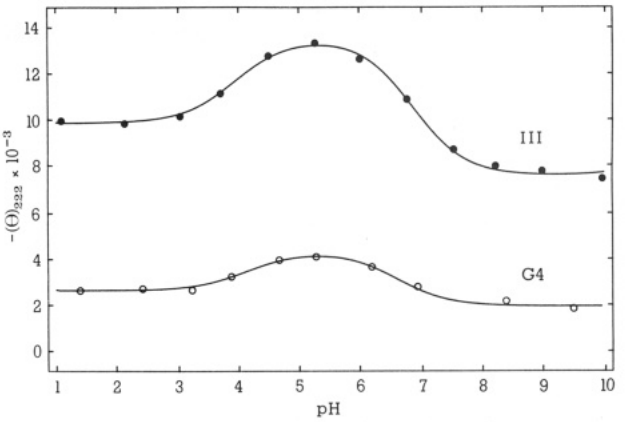

FIGURE 2: pH profiles of helicity at 3 °C as measured by $-[\theta]_{222}$ for reference peptides III and IIIA4G. $[\theta]_{222}$ is the mean residue ellipticity at 222 nm in degrees centimeter squared per decimole. See Materials and Methods for conditions.

Results

The pH profile of helicity at 3 °C (given by $-[\theta]_{222}$) is shown in Figure 2 for reference peptide III and for IIIA4G. The sequence of reference peptide III is acetyl-AETAAAK-FLRAHA-amide. There is a striking decrease in helicity at all pHs, caused by the replacement Ala-4 → Gly, but the pH profile of IIIA4G still shows the bell-shaped curve characteristic of the C-peptide helix. It is caused by the pH titration of Glu 2^- and His-12^+, as shown by replacement of these residues (Shoemaker et al., 1985, 1987a).

Figure 3 compares the pH profiles of helicity for five derivatives of reference peptide III with Ala → Gly at residue position 1, 4, 5, 6, or 13. The value of $-[\theta]_{222}$ at pH 5.3 is given in Table I for each peptide. It is apparent that the change in helicity for Ala → Gly is large at each interior residue position, 4, 5, or 6, and that there is a small increase in helicity as the substitution Ala → Gly is moved from position 4 toward position 6. The change in helicity is much smaller at either end residue, 1 or 13.

Discussion

Effect of the Replacement Ala → Gly at Interior Positions. The basic aim of this study was to find out if the replacement Ala → Gly causes a substantial change in the helicity of the

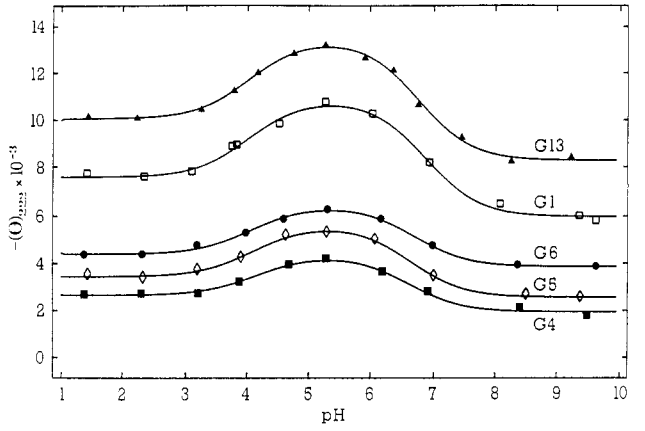

FIGURE 3: pH profiles of helicity at 3 °C for five derivatives of reference peptide III with the substitution of Ala → Gly at one of the five residue positions 1, 4, 5, 6, and 13.

Table I: Change in Helicity Caused by the Substitution Ala → Gly at One of Five Residue Positions in Reference Peptide III[a]

position of substitution	$-[\theta]_{222}$[b]
none[a]	13 200 ± 400
1	10 900 ± 500
4	4 200 ± 200
5	5 400 ± 250
6	6 500 ± 400
13	13 200 ± 100

[a] The sequence of reference peptide III (Shoemaker et al., 1987a) is CH_3CO-AETAAAKFLRAHA-NH_2. [b] Mean residue ellipticity of the peptide at 222 nm, 3 °C, pH 5.3. See Materials and Methods for conditions. The standard deviation is given in parentheses. From four to eight determinations were made, except for the peptide with G13, for which two determinations were made.

C-peptide helix and, if so, whether a similar change in helicity is found at each residue position. The results show a substantial change in helicity at the interior positions 4, 5, and 6. Since the results are similar at all three positions, we conclude that the change in helicity is caused primarily by the difference in s between Ala and Gly. The mutational replacement Gly → Ala in an α-helix within a protein has been found to increase the thermostability of the protein in three cases: λ repressor (Hecht et al., 1986), a neutral protease from *Bacillus stearothermophilus* (Imanaka et al., 1986), and phage T4 lysozyme (Matthews et al., 1987). The low value of s for Gly can be attributed both to the increased entropy of the unfolded peptide chain caused by greater rotational freedom about the N–C_α and C_α–CO bonds of a Gly compared to residues with bulkier side chains [cf. Creighton (1984), pp 162–170, and references cited therein] and to a favorable enthalpy of interaction of C_β with the peptide backbone [Gō et al., 1971; see p 3 of Scheraga (1973)]. A statistical survey comparing thermophilic and mesophilic variants of 70 proteins from 7 different protein families concluded that Ala → Gly is one of the most frequently used exchanges as proteins achieve increased thermal stability; moreover, this exchange is found most often in α-helices (Argos et al., 1979; Argos, personal communication).

We do not know what causes the small differences at the three interior positions. One possible explanation is that the flexibility introduced into the backbone by a glycine residue has an effect that depends on its position relative to a helix-stabilizing interaction such as the Glu-2–Arg-10 salt bridge. The results are sufficiently uniform at positions 4, 5, and 6 to justify proceeding with other residue substitutions at these positions in order to examine the correlation of P_α with the helix-stabilizing tendency.

Different Results Found at End Positions and at Interior Positions. A striking feature of the results is the small change in helicity caused by Ala → Gly at either end residue Ala-1 or Ala-13. A substantial change would be expected if formation of the C-peptide helix is a two-state reaction (see the following section) because of the sizable difference in s between Ala and Gly. The different results found at interior and end positions show either that formation of the C-peptide helix is not an all or none reaction, H ⇌ C (H = complete helix, C = random coil), or else that Gly has some unexplained helix-stabilizing effect when it is a terminal residue. If the reaction is all or none and if the relative helix-stabilizing effects of Ala and Gly are the same at any residue position, then the substitution Ala → Gly should change the overall equilibrium constant by the same factor at any residue position (see below).

There are two plausible explanations for the small change in helicity caused by Ala → Gly at a terminal position: each explanation requires that residues 1 and 13 are largely excluded from the helix, but one mechanism for exclusion is passive whereas the other is active. According to the passive mechanism, residues 1 and 13 are chiefly outside the helix because the ends of the helix are frayed: this causes a change in s at either end residue to have little effect on the helical content of the peptide. In the active mechanism, the Glu-2^-–Arg-10^+ salt bridge and the Phe-8–His-12^+ interaction serve as helix termination signals, so that residues 1, 2, and 13 are excluded from the helix.

Another formal possibility for the different results found at interior and end positions is that Gly has some helix-stabilizing effect when it is a terminal residue; no plausible basis for such an effect is known at present. In protein α-helices, Gly has a remarkably high probability of being the "C-cap" residue that is half-inside, half-outside, the helix at the C-terminal end (Richardson & Richardson, 1988; Schellman, 1980). Gly also has a higher than average probability of being the N-cap residue. There is an energetic reason for the striking preference of Gly for the C-cap position: it allows one extra intrahelix H bond to be made. As the chain turns away from the helix, Gly in the C-cap position assumes the α_L conformation. It then can H bond its NH group to the main chain CO group of residue C-cap–3, which otherwise would not be H bonded. To make way for the extra H-bond, residue C-cap+1 becomes H bonded to C-cap–4, which in a regular helix is H bonded to C-cap (Richardson & Richardson, 1988; Schellman, 1980). In peptide IIIA13G, Gly-13 could be the C-cap residue of the helix. Since the α-CO_2H group is blocked with an amide group, giving $CONH_2$, the NH of the terminal amide could H bond to a main-chain CO group: either to C-cap–4 when Gly-13 is in the α_L conformation or to C-cap–3 when Ala-13 is in the α_R conformation. Thus, the number of possible H bonds made would be the same with Gly-13 (α_L) or with Ala-13 (α_R). The contribution of residue 13 to $-[\theta]_{222}$ would, however, be opposite for Gly-13 in the α_L conformation and for Ala-13 in the α_R conformation. Since $[\theta]_{222}$ has exactly the same value in peptide III as in IIIA13G, it is probable that residue 13 is simply excluded from the helix, as discussed above. Thus, there is no apparent energetic reason why Gly should exert an extra helix-stabilizing effect at the C-terminal position in peptide IIIA13G. Nevertheless, it will be interesting to find out in future work if the same difference in helicity between interior and end positions is found for other amino acid replacements (Ala → X).

An NMR study of helix formation by a derivative of C-peptide (RN24), that differs from RN21 only in having succinyl-Ala in place of acetyl-Ala-1, shows that residues 1

and 13 are largely nonhelical (Osterhout et al., 1988) but does not distinguish between the two explanations above for exclusion of these residues from the helix. The stereochemistry of the Glu-2$^-$–Arg-10$^+$ interaction ensures that Glu-2 and residue 1 are outside the helix when the Glu-2$^-$–Arg-10$^+$ salt bridge is present. NMR data suggest that the Phe-8–His-12$^+$ ring interaction may also act as a helix stop (Osterhout et al., 1988).

Approximate Estimate of the Expected Change in Helicity. We can estimate the expected change in helicity for a simple model in which the formation of a short helix is a two-state reaction and the apparent equilibrium constant K^* is the product of the s values for all residues times some constant. Then $K^*_G/K^*_A = s_G/s_A$. This model is known to be too simple to represent the effect of Ala → Gly at end residues, as discussed above. To compute the expected effect at an interior position by this model, we take K^* to be $f/(1-f)$, where f is the fraction of helix given by $([\theta] - [\theta]^0)/([\theta]^{100} - [\theta]^0)$ and $[\theta]^0$ and $[\theta]^{100}$ refer to values of $[\theta]_{222}$ for 0% and 100% helix, respectively. The value of $[\theta]^0$ at 3 °C is +2500 ± 500 deg cm^2 dmol^{-1} (Shoemaker et al., 1988), and the value of $[\theta]^{100} - [\theta]^0$ is estimated at −29 000 ± 1000 deg cm^2 dmol^{-1} from measurements of adding largely nonhelical S-peptide(1–15) to folded S-protein, the end product being completely folded RNase S (Mitchinson & Baldwin, 1986). Then the value of K^*_G/K^*_A measured from data in Table I is 0.37, whereas the estimated value based on s_G/s_A is 0.51/1.08 = 0.47 at 3 °C. Thus, the observed change in C-peptide helicity for Ala → Gly is estimated correctly to a first approximation by this oversimplified model.

Use of the C-Peptide Helix To Measure the Relative Helix-Stabilizing Tendencies of Different Amino Acids. This study indicates that it should be possible to use the C-peptide system to correlate P_α with the relative helix-stabilizing tendencies of different amino acids. Such a study may uncover reasons contributing to the imperfect correlation between values of s given by the host–guest method and P_α [see p 331 of Creighton (1984)]. Deviations from this correlation are likely to arise for interesting reasons. Maxfield and Scheraga (1975) pointed out that Glu is one of the most commonly found amino acids in protein helices although the s value of Glu$^-$ indicates that it is an indifferent helix-former. They found statistical evidence suggesting that Glu$^-$ can stabilize helices through salt bridge formation, and later direct evidence for helix stabilization by (i, i + 4) Glu$^-$–Lys$^+$ salt bridges was found by Marqusee and Baldwin (1987). It is possible that other types of side chain interactions, such as hydrophobic interactions, influence both the results of host–guest experiments and the frequencies of amino acids in protein helices.

As regards the second aim of this work, to explore the feasibility of using C-peptide to study position-dependent effects of different amino acids, especially at helix termini, we conclude that it will be difficult to study amino acid replacements at the N- or C-terminus of C-peptide because of the strong possibility that terminal residues are largely excluded from participation in helix formation.

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Registry No. RNase C-peptide, 3150-30-9; peptide III, 102636-66-8; Gly^1-peptide III, 118460-29-0; Gly^4-peptide III, 118460-30-3; Gly^5-peptide III, 118460-31-4; Gly^6-peptide III, 118460-32-5; Gly^{13}-peptide III, 118460-33-6.

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