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Stabilization of α-helical structures in short peptides via end capping

(peptide synthesis/protein folding/circular dichroism)

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ABSTRACT **The α-helix-stabilizing effect of different amino acid residues at the helical termini of short peptides in aqueous solution has been determined. Several dodecapeptides containing alanine, asparagine, aspartate, glutamine, glutamate, and serine at the amino terminus and arginine, lysine, and alanine at the carboxyl terminus were synthesized, and the α-helical content of each peptide was measured by using circular dichroism spectroscopy. The trend in α-helix-inducing ability of these amino acids was found to be as follows: aspartate > asparagine > serine > glutamate > glutamine > alanine at the amino terminus and arginine > lysine > alanine at the carboxyl terminus. Our results agree with the Presta and Rose hypothesis [Presta, L. G. & Rose, G. D. (1988) *Science* 240, 1632–1641] on the role of end capping in helix stabilization.**

Designed peptides and proteins that fold into predetermined secondary structures are valuable tools in protein structure–function studies (1–3). The α-helix, first proposed as a model structure by Pauling *et al.* (4) and experimentally studied by Perutz (5) is a landmark in protein secondary structures. Empirical predictions (6, 7) and experimental studies (8–18) have provided a wealth of information on helix formation in peptides and polypeptides. These studies showed the variations in helix propensities of the amino acids (8–12), as well as the helix-stabilizing role of charged side-chain–helix dipole interactions (13), salt bridges (14), disulfide linkages (15), hydrophobic periodicity (16), aromatic interactions (17), and metal chelation (18). Most of the experimental work involves amino acid substitutions in the middle of the helix.

In an α-helix, NH donors of the first four residues and CO acceptors of the last four residues lack intrahelical hydrogen-bond partners. Presta and Rose (19) hypothesized that a necessary condition for helix formation is the presence of residues flanking the helix termini that have side chains to supply hydrogen-bond partners for unpaired main-chain NH and CO groups. Richardson and Richardson (20), by surveying several proteins of known structures, showed that at the amino termini of helices, there is a preponderance of amino acids with side chains that could hydrogen-bond with the free NH groups of the helix. Gierasch and coworkers (21) have shown that side-chain–backbone hydrogen bonding, as proposed by Presta and Rose, may also stabilize helix formation in peptides. Site-directed mutagenesis studies involving the amino-terminal residues of α-helices in native proteins have shown the significance of such hydrogen bonding in protein stability (22, 23).

We decided to test the Presta and Rose hypothesis in a peptide designed to fold into an isolated α-helix (24). The rationale for the design is as follows. (*i*) We chose a short α-helical segment (12 residues) resembling most α-helices found in native proteins. Further, we expected the capping effects to be more prominent in a short peptide. (*ii*) We introduced a proline and a glycine, amino acids traditionally known to be helix breakers, at positions 2 and 11, respectively, to demarcate the helical ends (25). We also hoped that the presence of the helix breakers might enable the side chains of the end residues to turn around and hydrogen-bond with the free NH and CO groups at the helical termini. (*iii*) The presence of a glutamate at position 4 and a lysine at position 7 increases solubility of the peptide and may also stabilize the helix by salt bridge formation (14). (*iv*) The rest of the amino acids were chosen to be alanine, which has a high tendency to form α-helices (26). (*v*) The respective positively and negatively charged side chains of arginine at the carboxyl end and aspartate at the amino end are expected to interact favorably with the helical dipole and stabilize the α-helix (13). (*vi*) The negatively charged carboxyl group of Arg-12 is amidated to eliminate repulsive interactions between itself and the negative pole of the helical dipole. (*vii*) The α amino group of residue 1 was not acylated to prevent such an amide carbonyl from hydrogen bonding with the free NH groups at the amino terminus. The sequences of our model peptide and its variants are given in Table 1.

Table 1. Amino acid sequences of peptides

	Sequence											
Peptide	1	2	3	4	5	6	7	8	9	10	11	12
I	D	P	A	E	A	A	K	A	A	A	G	R-amide
II	A											
III	N											
IV	Q											
V	S											
VI	E											
VII												K-amide
VIII												A-amide
IX		G										
X											P	

The single-letter code for amino acids is used.

MATERIALS AND METHODS

Peptide Synthesis. The peptides were synthesized as their carboxamides by using 5-[4-(9-fluorenylmethoxycarbonyl)-aminomethyl-3,5-dimethoxyphenoxy)] valeric acid (PAL) resin from Milligen/Biosearch (Novato, CA) on a Milligen/Biosearch 9050 peptide synthesizer using 9-fluorenylmethoxycarbonyl (Fmoc) chemistry and benzotriazolyl *N*-oxytris-(dimethylamino)phosphonium hexafluorophosphate (BOP)/1-hydroxybenzotriazole (HOBT) activation. The peptides were cleaved from the resin with 90% trifluoroacetic acid/5% thioanisole/3% ethane dithiol/2% anisole at room tempera-

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ture for 6 hr. The peptides were precipitated from ice-cold diethyl ether.

Purification. The peptides were purified to homogeneity on a reversed-phase C_{18} HPLC column (Rainin, Woburn, MA), using water and acetonitrile/0.1% trifluoroacetic acid as the solvent system. A gradient of 0–20% acetonitrile in 30 min gave efficient separation. The detector was set at 215 nm. The peptide purity was checked by analytical HPLC, and the primary structure was confirmed by quantitative amino acid analysis done at the Protein Structure Laboratory at the University of California, Davis.

Circular Dichroism (CD) Measurements. CD spectra were recorded on a Jasco 600 spectropolarimeter equipped with a Lauda K-2/R temperature controller. A total of four scans were averaged to obtain each spectrum. All CD studies were done in 10 mM KF, using a 10-mm-path-length cell. The pH was adjusted to 7.0 with HCl and NaOH. The peptide concentration in CD experiments was 17 μM, except during determination of concentration dependence. The peptide concentrations were determined by quantitative amino acid analysis done on a Beckman 6300 high-performance amino acid analyzer, with an error estimate of ±2%.

RESULTS AND DISCUSSION

The helix content was measured on 17 μM solution of each peptide by CD at low temperature (0°C) in 10 mM KF at pH 7. The peptides show characteristic CD spectra (27, 28), indicating a mixture of α-helix and random coil with a negative band centered at 222 nm (Fig. 1). Helical content is taken directly proportional to the mean residue ellipticity at 222 nm $[\theta]_{222}$. One hundred percent helicity was estimated by using the formula $^{max}[\theta]_{222} = -40{,}000 \times [1 - (2.5/n)]$, where n = number of amino acid residues (29). According to this method, the mean residue ellipticity at 222 nm for a 12-residue peptide in a completely helical conformation is −31,500. α-Helicity was determined to be independent of peptide concentration in the range of 10–300 μM, showing that these peptides are monomeric in solution (data not shown). Table 2 shows the α-helical content of the peptides. Mean residue ellipticity $[\theta]_{222}$ is calculated by dividing molar ellipticity by 12 because all peptides contain 12 amino acids. Percentage helicity = $100 \times ([\theta]_{222}/-31{,}500)$.

Table 2. α-Helical content of peptides

Peptide	$-[\theta]_{222}$,* deg·cm²·dmol⁻¹	Helicity,† %
I	7900	25.1
II	760	2.4
III	7700	24.4
IV	1500	4.8
V	6800	21.6
VI	2700	8.6
VII	6400	20.3
VIII	5200	16.5
IX	5700	18.1
X	1800	5.7

*Mean residue ellipticity $[\theta]_{222}$ has been determined from the CD spectra of 17 μM solution of peptides in 10 mM KF at 0°C and pH 7.

†Percentage helicity has been calculated as $100 \times ([\theta]_{222}/^{max}[\theta]_{222})$. $^{max}[\theta]_{222} = -40{,}000\ [1 - (2.5/n)]$, where $n = 12$.

Amino Terminal Capping. The potential α-helix-inducing effect of amino acids aspartate, alanine, asparagine, glutamine, glutamate, and serine by amino-terminal capping was determined by synthesizing peptides I–VI, which contain these amino acids at position 1. The CD spectra of these peptides are shown in Fig. 1. Peptide I with aspartate at position 1 folds into a partial α-helix. The CD spectra of peptide 1 in the presence of increased concentrations of trifluoroethanol show a gradual increase in α-helical content (Fig. 1*B*). Substitution of Asp-1 by alanine (peptide II) causes a 10-fold decrease in helicity. Peptide II behaves like a random coil and gave a CD spectrum typical of a random-coil structure (28). It is interesting to see that a single-amino acid substitution at the amino end has such a profound effect on the secondary structure of the peptide as a whole. The side chain of alanine, unlike that of aspartate, cannot hydrogen-bond with the free NH groups at the amino terminus of the helix, and this property may explain the drastic difference in helicity of the two peptides. Asn-1 substitution (peptide III)

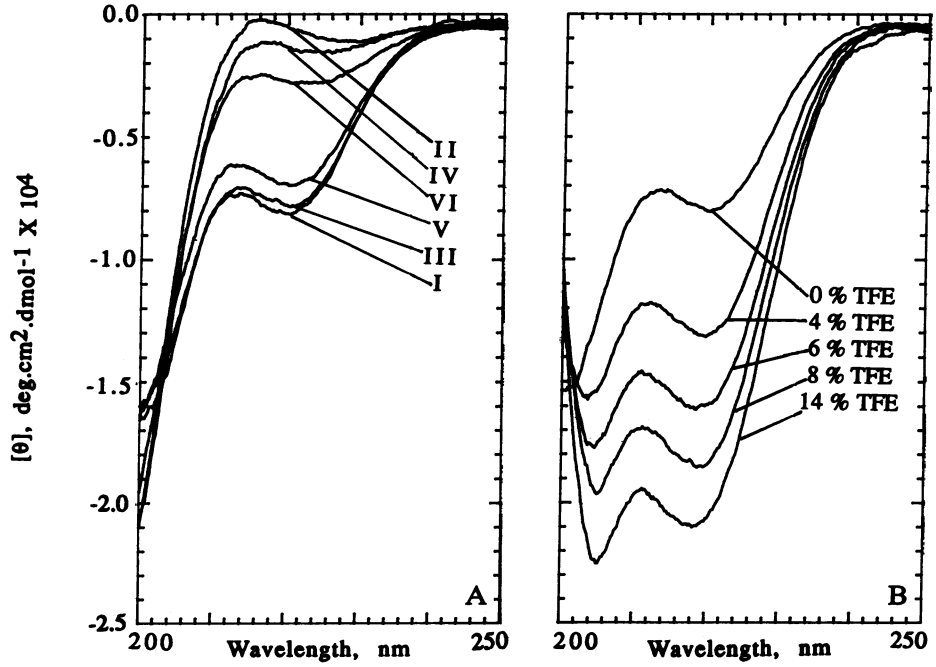

FIG. 1. (*A*) CD spectra of 17 μM solution of peptides I–VI at 0°C in 10 mM KF. (*B*) Change in CD spectrum of 17 μM solution of peptide I at 0°C in 10 mM KF at 0, 4, 6, 8, and 14 mol percentage trifluoroethanol (TFE).

exhibits similar α-helicity as peptide I, although slightly lower. The decrease, although very slight, is reproducible and can be explained by the fact that while asparagine side chain can effectively form a hydrogen bond, it cannot interact favorably with the helical dipole and stabilize the helix, as is possible with aspartate. Further, the carboxylate group of aspartate can possibly form two hydrogen bonds with two NH groups, whereas asparagine can only form one hydrogen bond. Substitution of asparagine by serine (peptide V) shows a small decrease in helicity. This decrease could be due to the fact that although both asparagine and serine side chains could form hydrogen bonds, asparagine may have better hydrogen-bond orientation than serine. The results of Asn-1 → Ser substitution, while in complete agreement with Richardson and Richardson's statistical correlation (20), differ from the results of Thr → Asn substitution experiments in Barnase (22) and T4 lysozyme (23). In these proteins, tertiary interactions involving other residues are believed to stabilize the α-helix. Asp-1 → Glu substitution (peptide VI) results in a 3-fold decrease in helicity. This result can be rationalized by the fact that the longer side chain of glutamate does not have the right geometry to form hydrogen bonds as aspartate does but still might be able to compensate for the helical dipole. The Asp-1 → Gln substitution (peptide IV) resulted in an even larger decrease (5-fold) in helicity. The helix-inducing potential of these amino acids at position 1 was found to be in the following order: aspartate > asparagine > serine > glutamate > glutamine > alanine. These results are completely consistent with the hypothesis that side-chain–main-chain hydrogen bonds at the amino terminus are helix stabilizing.

Carboxyl-Terminal Capping. The potential carboxyl-terminal-capping efficiency of amino acids arginine, lysine, and alanine was determined by synthesizing peptides I, VII, and VIII, having these amino acids, respectively, at position 12 (Table 2). Replacing arginine with lysine at position 12 shows a decrease in α-helicity. The guanido group of arginine, with the capability of forming multiple hydrogen bonds and interacting favorably with the helical dipole, can stabilize the helix better than lysine. Peptide VIII with Ala-12 showed an even lower value as compared to peptide VII with Lys-12. This result is, indeed, expected because the side chain of alanine can neither form hydrogen bonds nor interact with the helical dipole. The order of amino acids preferable at the carboxyl end is clearly arginine > lysine > alanine.

Role of Helix Breakers. Proline, due to lack of an NH group, can eliminate one of the nonhydrogen-bonded NH groups at the amino terminus. Richardson and Richardson (20) have shown that in native proteins, side-chain functional groups are frequently seen to hydrogen-bond with the backbone NH of N2 and especially N3 but almost never to N1. Hence, proline should be best-suited at position 2. In addition, proline, by virtue of its cyclic ring structure, makes it energetically unfavorable for the preceding residue to be in α-helical conformation (25, 30). Further, the residues at the helical termini in proteins have been shown to adopt a conformation nonconsistent with the usual α-helical ϕ,ψ angles (19). However, a study by MacArthur and Thornton (31) has found a small but significant (<10%) fraction of Xaa-Pro combinations where residue Xaa does adopt a helical conformation (31). Replacing Pro-2 by glycine (peptide IX) results in a decrease in α-helicity (Table 2), showing that proline is better than glycine at position 2.

The Gly-11 → Pro substitution showed a 4-fold decrease in α-helicity (Table 2). Such a large decrease is, indeed, expected because of the inability of proline to continue α-helical hydrogen bonding due to the lack of an NH group that can participate in the internal hydrogen bonding. Similar results were obtained by Baldwin and coworkers (32) by introducing proline in a 13-residue peptide. Thus, proline is the residue of choice at position 2 and glycine is the residue of choice at position 11 as helix breakers. Our results agree with Richardson and Richardson's statistical correlation (20). Richardson and Richardson observed a preference of 2.6:1 for proline at N1 and a preference of 3.9:1 for glycine at the carboxyl-termini cap. The exact structural characteristics of these residues at the ends of helices are uncertain and need to be determined.

Temperature Studies. The CD spectra of 17 μM solutions of each peptide in 10 mM KF at pH 7 were monitored as a function of temperature in the range of 0–70°C. The effects of temperature on the mean residue ellipticity of peptides I and II are shown in Fig. 2. It is worth noting that peptide I with

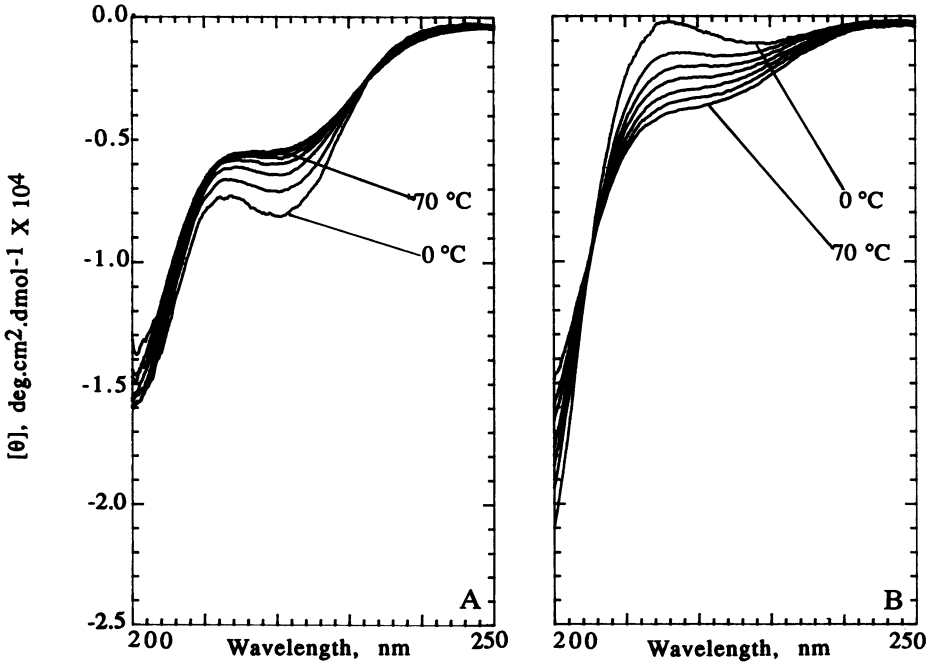

FIG. 2. Change in CD spectra of 17 μM solution of peptides at 0°C in 10 mM KF as a function of temperature (0–70°C). (*A*) Peptide I. (*B*) Peptide II.

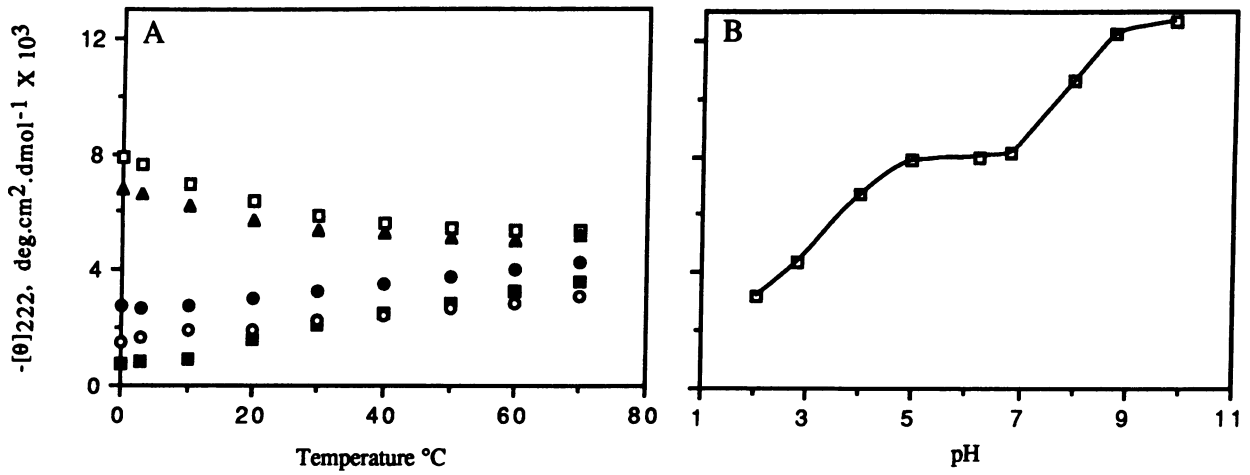

FIG. 3. (*A*) Change in mean residue ellipticity $-[\theta]_{222}$ for peptides I (□), II (■), IV (○), V (▲), and VI (●) as a function of temperature. (*B*) Change in mean residue ellipticity $-[\theta]_{222}$ of peptide I as a function of pH at 0°C.

an aspartate at the amino end does not exhibit an isodichroic point, characteristic of a two-state system. It is conceivable that the possible hydrogen bonding between aspartate and the amino-terminal NH groups results in the formation of a relatively stable third structure. Peptide II with an alanine at the amino end does show an isodichroic point at near 207 nm, which reflects the temperature dependence of the CD of a random coil (33). The effects of temperature on peptide variants are shown in Fig. 3*A*. The data indicate that at high temperature all the peptides unfold into a similar structure. As the temperature is lowered, the helix-forming peptides (I, III, V, VII, VIII, and IX) show a decrease in $[\theta]_{222}$, indicative of cooperative folding into an α-helix. The poor helix formers (II, IV, VI, and X) show an increase in $[\theta]_{222}$, characteristic of a random coil thermal behavior (Fig. 3*A*). Similar results have been seen in other systems by Yang and Doty (34) and Baldwin and coworkers (35).

pH Studies. Change in helicity of a 17 μM solution of peptide I was monitored as a function of pH (Fig. 3*B*). An increase in α-helicity is observed between pH 2–5 and 7–9. The increase in the pH range 2–5 is due to titration of side chains of aspartate and glutamate. The carboxyl anion of Asp-1 can interact favorably with the helical dipole and that of glutamate can form a salt bridge with lysine, both factors contributing to helical stability. The second increase in helical content in the pH range 7–9 is from the removal of a positive charge from the amino group of Asp-1. Removing a positive charge from the amino terminus eliminates an unfavorable charge–dipole interaction.

Conclusion. Our results clearly show that it is possible to induce short peptides (12 residues) to adopt α-helical structures in aqueous solution. Further, these data provide experimental evidence for the Presta and Rose hypothesis that states that a necessary condition for helix formation is the presence of amino acid residues flanking the helix termini whose side chains can hydrogen bond with the first four NH groups at the amino end and the last four CO groups at the carboxyl end, which lack intrahelical hydrogen-bond partners (19). Removal of hydrogen-bonding side chains from the amino-terminal residue has a profound effect on the helical structure of the whole molecule. The results also suggest that the amino acid preferences for specific locations at the ends of α-helices, observed by Richardson and Richardson (20), reflect elements of structural stability. Richardson and Richardson observed a 3.5:1 preference for asparagine at the N-cap position, whereas aspartate is preferred 2.1:1. We find that aspartate at the N-cap position is slightly better than asparagine and believe that this is due to its ability to compensate for the helical dipole in addition to participating in hydrogen bonding. Thus, in isolated peptides, aspartate at the amino end and arginine at the carboxyl end are excellent helix stabilizers. Both of these residues might function by capping the helix ends and favorably interacting with the helical dipole.

Note. After the conclusion of our work (24), we learned that Lyu *et al.* obtained related amino end-capping results (36).

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