PROTEINS: Structure, Function, and Genetics 10:130–139 (1991)

Side Chain–Backbone Hydrogen Bonding Contributes to Helix Stability in Peptides Derived From an α-Helical Region of Carboxypeptidase A

Martha D. Bruch, Madan M. Dhingra, and Lila M. Gierasch
Department of Pharmacology, The University of Texas Southwestern Medical Center, Dallas, Texas 75235-9041

ABSTRACT **Recently, Presta and Rose proposed[1] that a necessary condition for helix formation is the presence of residues at the N- and C-termini (called NTBs and CTBs) whose side chains can form hydrogen bonds with the initial four amides and the last four carbonyls of the helix, which otherwise lack intrahelical hydrogen bonding partners. We have tested this hypothesis by conformational analysis by circular dichroism (CD) of a synthetic peptide corresponding to a region (171–188) of the protein carboxypeptidase A; in the protein, residues 174 to 186 are helical and are flanked by NTBs and CTBs. Since helix formation in this peptide may also be stabilized by electrostatic interactions, we have compared the helical content of the native peptide with that of several modified peptides designed to enable dissection of different contributions to helix stability. As expected, helix dipole interactions appear to contribute substantially, but we conclude that hydrogen bonding interactions as proposed by Presta and Rose also stabilize helix formation. To assist in comparison of different peptides, we have introduced two concentration-independent CD parameters which are sensitive probes of helix formation.**

Key words: **α-helix, side chain–backbone hydrogen bonding, helix dipole, circular dichroism, carboxypeptidase A**

INTRODUCTION

The helix hypothesis of Presta and Rose[1] states that α-helix formation is favored by hydrogen bonding interactions involving the residues at the N- and C-termini of the helical segment. Specifically, Presta and Rose propose that a necessary condition for helix formation is the presence of N- and C-terminal residues that have side chains capable of forming hydrogen bonds with the initial four amides and final four carbonyls, groups which otherwise lack intrahelical hydrogen bonding partners. Residues whose side chains can hydrogen bond to amide groups, e.g., Asn, Asp, Gln, Glu, Ser, Thr, and His, can function as NTBs, whereas CTBs are comprised of residues that can hydrogen bond to carbonyl groups, e.g., Asn, Arg, Gln, Lys, Ser, Thr, and His. One prediction of this model is that hydrogen bonding residues, referred to as NTBs and CTBs, stabilize helix formation, and, therefore, that isolated peptides which are flanked by NTBs and CTBs will have a high probability to fold into helices in aqueous solution.

Although the importance of electrostatic interactions for helix stability has been demonstrated for several different peptides,[2–7] there have been very few experimental tests of the helix hypothesis. Fairman et al.[5] compared helix stability in a C-peptide analogue with an amide C-terminal blocking group, which can hydrogen bond to the backbone, and one with a methyl ester at the C-terminus, which would lack this potential stabilizing effect. No significant difference in helicity was found. On the other hand, Serrano and Fersht[8] attributed changes in the folding energetics of barnase mutants to the removal of N-terminal hydrogen-bonding interactions of the type described by Presta and Rose. In the present study, we have used circular dichroism (CD) spectroscopy to assess the relative contributions to helix stability of NTB and CTB hydrogen bonding interactions in a synthetic peptide corresponding to residues 171–188 of the protein carboxypeptidase A (Table I). The crystal structure of this protein reveals a helix encompassing residues 174–186,[9] so the synthetic peptide analyzed contains a region (in peptide numbering, residues 4–16) which is helical in the native protein. Inspection of the sequence shows that the helical region is flanked by residues which can serve as NTBs (Asn, Ser, Glu) and CTBs (Asn, His), and the helix hypothesis predicts that

Received June 25, 1990; revision accepted November 2, 1990.

Address reprint requests to Dr. Lila M. Gierasch, Department of Pharmacology, The University of Texas Southwestern Medical Center, 5323 Harry Hines Blvd., Dallas, TX 75235-9041.

Present address for M.D. Bruch: Department of Chemistry and Biochemistry, University of Delaware, Newark, DE 19716.

Present address for M.M. Dhingra: Tata Institute of Fundamental Research, Chemical Physics Group, Homi Bhabba Road, Bombay-400005, India.

TABLE I. Peptides Studied*

Protein numbering	171			175				180				185				
Peptide numbering	1			5				10				15				
CPA	Ac-N	S	E	V	E	V	K	S	I	V	D	F	V	K	N	H	G	N-Amide
MCPA1	Ac-N	S	Q	V	E	V	K	S	I	V	D	F	V	K	N	S	G	N-Amide
MCPA2	Ac-A	A	A	V	E	V	K	S	I	V	D	F	V	K	N	A	A	A-Amide

*Residues altered from the native are underlined.

the isolated peptide, CPA, will fold autonomously into a helix.[1,*] However, this peptide also has charged groups near the N- and C-termini (Glu-3 and His-16, respectively) which can contribute to helix stability through interactions with the helix dipole. Furthermore, this peptide has the potential to form both $i/i+3$ and $i/i+4$ salt bridges between Asp-11 and Lys in positions 14 or 7, respectively, as well as an $i/i+4$ salt bridge between Glu-3 and Lys-7.

To dissect the contributions to helix stability from these different potential sources, we have compared the folding behavior of the native peptide to that of two modified peptides, MCPA1 and MPCA2, whose sequences are shown in Table I. All three peptides were acetylated and amidated to remove the destabilizing effects of the charged N- and C-termini, and they all retain the potential to form salt bridges between Asp and Lys. However, in the peptide MCPA1, the charged groups at the N- and C-termini, Glu-3 and His-16, were changed to Gln and Ser, respectively. This removes the potential interactions between the charged groups and the helix dipole but does not affect the ability of these residues to serve as NTBs and CTBs. Therefore, differences in the helical content of CPA and MCPA1 will reflect primarily contributions to helix stability from the charged residues, presumably with the helix dipole. To determine the contribution from NTBs and CTBs, the three N- and C-terminal residues in MCPA1 were replaced by alanines, which are neither charged nor participate in side chain hydrogen bonding, in the peptide MCPA2. Differences between MCPA1 and MCPA2 can thus be attributed to hydrogen bonding interactions, and comparison of the helix stability of these two peptides provides a direct test of the helix hypothesis.

It should be noted that a comparison of MCPA1 and MCPA2 is complicated by the different inherent propensities for α-helical conformations associated with the residues in these two peptides. Both Chou/Fasman rules for secondary structure prediction[10] and host/guest determination of helical propensities[11] indicate that alanine has a high tendency to adopt a helical conformation. The helix stabilizing effect of alanine was further demonstrated by the high helical content observed for alanine based peptides.[12,13] Therefore, the peptide MCPA2 is predicted to have a higher inherent tendency to fold into a helix than MCPA1 or CPA, irrespective of any electrostatic or hydrogen bonding effects. In fact, Chou/Fasman rules[10] yield average helical propensities of 1.02, 0.98, and 1.19 for the peptides CPA, MCPA1, and MCPA2, respectively, and any comparison among these peptides must take these differences into account.

Helix formation and propagation within a given peptide is readily monitored by CD spectroscopy since the band at 222 nm is due almost exclusively to α-helical structures.[14] However, distinguishing small differences between the helical content of *different* peptides is more difficult since the relative intensities of their CD spectra are dependent on the peptide concentration. Hence, errors in peptide concentration will lead to significant errors in the amount of helix determined from the CD intensity at 222 nm. We avoid this problem by introducing two new parameters, R1 and R2, each of which is a ratio of intensities in the CD spectrum. These parameters are independent of the peptide concentration but are sensitive measures of the amount of helix present in a peptide which is in equilibrium between α-helical and random coil conformations. Therefore, these parameters are quite useful for comparison of the helical content of different peptides, especially when the overall amount of helix formation is small. Our results show that there is a distinct difference between the helical content of the native CPA peptide and MCPA1, and this argues that the helix dipole plays a dominant role in helix stability, as expected from previous work on other helical peptides. However, there is also a significant difference between the helical content of MCPA1 and MCPA2, and this demonstrates that hydrogen bonding between the side chains and the peptide backbone also contributes to helix stability.

EXPERIMENTAL SECTION

Synthesis

Peptides were synthesized by solid phase methods using a Milligen 9050 peptide synthesizer and Applied Biosystems peptide synthesizer model 430A.

*The lysine residue at position 191 of carboxypeptidase was included as a CTB by Presta and Rose. We have studied most extensively the 18-residue peptide *without* this residue. However, a peptide with the native sequence including this lysine (i.e., 21-residue peptide) was synthesized and found *not* to differ significantly in helix content (including pH dependence) from the 18-residue peptide.

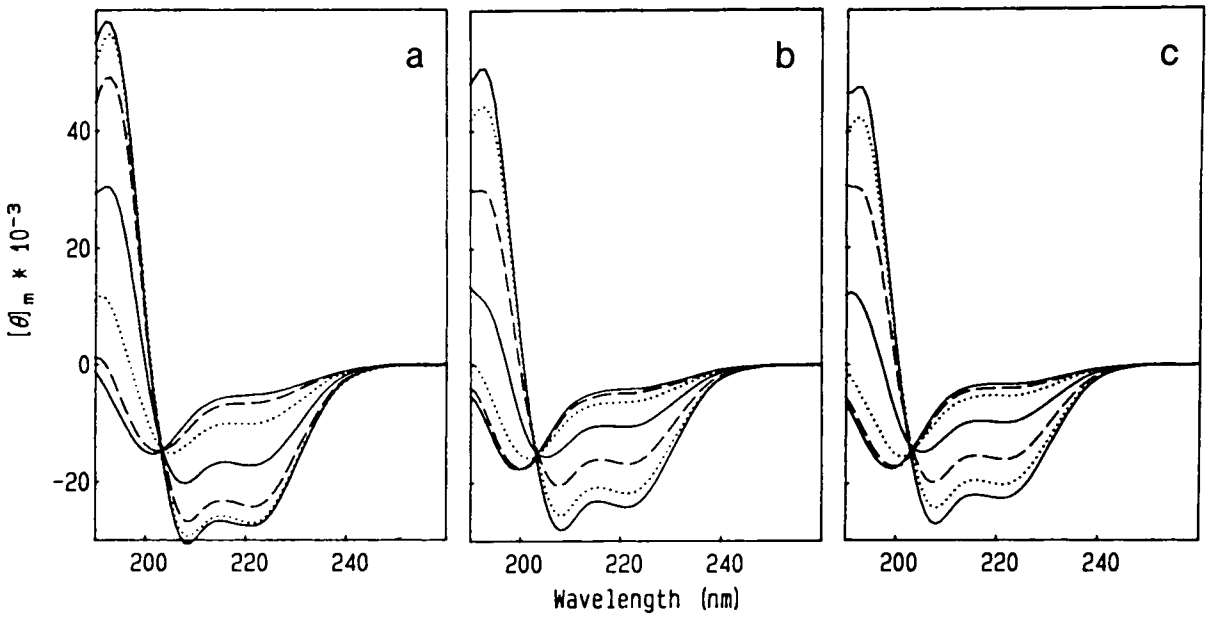

Fig. 1 CD spectra as a function of TFE content for (**a**) CPA, (**b**) MCPA1, and (**c**) MCPA2 at 25°C. The percentages of TFE used (by volume) were 0% (solid line), 5% (dashed), 10% (dotted), 15% (solid), 20% (dashed), 30% (dotted), and 50% (solid).

The peptides were purified by preparative high-performance liquid chromatography (HPLC) using a Vydac C_{18} column which was 28 cm long and had an internal diameter of 2 cm. The purity and identity of the peptides were checked by analytical HPLC, amino acid analysis, and fast atom bombardment mass spectrometry.

Circular Dichroism

All CD spectra were obtained on an AVIV model 60DS CD spectrometer. The temperature was regulated by a Hewlett Packard 89100 A temperature controller. The peptide concentrations were determined by quantitative amino acid analysis. The peptide concentration ranged from 5 to 30 μM; no changes were observed in the CD spectra over this concentration range. The pH was maintained by phosphate buffer (4.5 mM). All spectra were obtained in a 5 mm cell and were the average of three consecutive scans with a total duration of 9 sec at each nm from 260 to 190 nm. All spectra were baseline corrected and smoothed.

RESULTS AND DISCUSSION

TFE Titrations

The CD spectra of all three CPA peptides in aqueous buffer at 25°C (Fig. 1) exhibit a small, but distinct, minimum at 222 nm and a larger minimum at approximately 200 nm, and this shape is indicative of a mixture of α-helical and random coil conformations.[14] As an initial probe of the ability of these peptides to adopt a helical conformation, trifluoroethanol (TFE) was added incrementally to the aqueous solution of each peptide. Addition of TFE to aqueous peptide solutions has been shown to stabilize α-helix formation, but not indiscriminately.[15,16] For each of the three peptides, the CD spectra as a function of TFE exhibit an isodichroic point at 203 nm, indicating that the structure of each peptide can be described as a two-state equilibrium between a random coil and an α-helix. Furthermore, the shape of the CD spectra obtained in a solution of 50% TFE (by volume) is indicative of a high population of helical conformation for all three peptides. Since TFE has been shown to be a good probe for the *potential* of peptides (or regions of peptides) to adopt a helical conformation,[15,16] the high population of helical conformation observed in 50% TFE demonstrates that each peptide has the ability to adopt preferentially an α-helical conformation. Although no information can be obtained from the CD spectra regarding the length or position of the α-helix in each peptide, the high content of α-helix achieved at 50% TFE suggests that, in each case, the helix can potentially include nearly all of the residues of the peptide. This conclusion is supported by preliminary data from two-dimensional nulear magnetic resonance (2D NMR) experiments (Bruch, Dhingra, and Gierasch, unpublished results).

Comparison among the different peptides is facilitated by replotting the CD spectra at different TFE content as shown in Figure 2. At each point in the TFE titration, the CD spectrum for CPA (solid line) has several qualitative features which indicate the CPA has a higher helical content than the other two peptides. First, the intensity at 222 nm, $[\theta]_{222}$, which

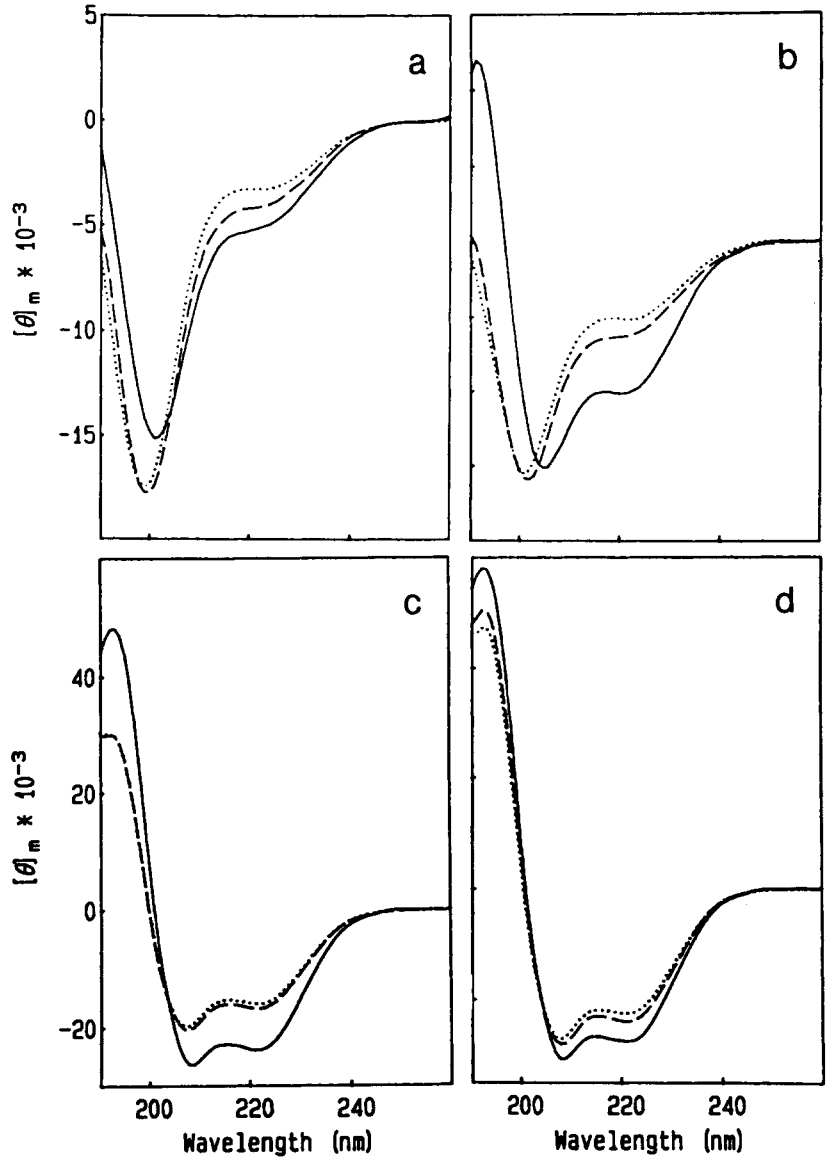

Fig. 2. CD spectra for the three peptides: CPA (solid line), MCPA1 (dashed), MCPA2 (dotted) at pH 5.3, 25°C in (**a**) 0% TFE, (**b**) 10% TFE, (**c**) 20% TFE, and (**d**) 50% TFE.

is a good measure of α-helical content, is larger in CPA than in the other peptides. In addition, the position of the low wavelength minimum, which varies from 195 to 210 nm as the equilibrium is shifted from random coil to α-helical conformation, occurs at a higher wavelength in the CD spectra of CPA than those of the other two peptides. These observations suggest that CPA has a higher helical propensity than either MCPA1 or MCPA2. The CD spectra of MCPA1 and MCPA2 obtained in 0 or 10% TFE exhibit similar, albeit smaller, qualitative differences than those observed between CPA and MCPA1. However, as discussed below, limitations of existing methods to analyze CD spectra for low α-helical content make it difficult to demonstrate *quantitatively* that these *qualitatively* apparent spectral differences truly reflect conformational differences between the peptides MCPA1 and MCPA2.

One method currently used to quantitate helical content is based on the mean residue ellipticity at 222 nm, $[\theta]_{222}$, since this band is due nearly exclusively to α-helical structures. According to this method, the mean residue ellipticity at 222 nm for a 18-residue peptide which is 100% helical is approximately −30800.[17] Due to variabilities in the exact nature of a random coil conformation, it is difficult to pinpoint the expected value for $[\theta]_{222}$ in a peptide which is 100% random.[14] Since addition of the de-

TABLE II. TFE Titration of CPA Peptides at pH 5.3: Estimation of % α-Helix

% TFE	CPA $[\theta]_{222}$*	% α-helix shape†	MCPA1 $[\theta]_{222}$	% α-helix shape	MCPA2 $[\theta]_{222}$	% α-helix shape
0	17	30	14	25	11	20
5	21	30	16	25	13	20
10	33	45	20	28	16	28
15	57	68	34	45	30	45
20	78	93	56	70	52	70
30	84	100	61	80	66	75
50	86	98	76	87	69	80

*% α-helix determined from $[\theta]_{222}$ according to Eq. (1).[17] Estimated relative error of ±5% for CPA, MCPA2. Estimated relative error of ±10% for MCPA1.

†These results are obtained from curve fitting to polylysine reference spectra.[18] Although results for an individual trial are rounded to the nearest 5%, the values in the table are not necessarily multiples of 5% due to averaging between two or more trials. Estimated absolute error of ±3 for all peptides.

naturant guanidinium hydrochloride suggests that the ellipticity at 222 nm is near zero for these peptides when the helix is totally destabilized, zero was used as the value for $[\theta]_{222}$ corresponding to 0% helix. Therefore, the percentage of α-helix is given by

$$\% \alpha = (100)([\theta]_{222})/(-30800) \quad (1)$$

Another method for quantitation of the amount of helix present is based upon the shape, not the intensity, of the CD spectrum. This method estimates the amount of helix present by curve-fitting the experimental CD spectrum to reference spectra for α-helix, β-sheet, and random coil conformations of polylysine.[18]

Both of these quantitative methods are subject to a significant amount of uncertainty for different reasons: The former method is dependent upon peptide concentration, which is difficult to measure with high precision, whereas the latter method assumes that the shape of the CD spectrum for a short peptide is the same as that observed for a homopolymer. The problems associated with quantitation of the amount of helix present are magnified when different peptides are compared. Unlike comparisons within a given peptide, comparisons between different peptides require the determination of the concentration of different peptide solutions, and this can lead to systematic errors in the CD intensities. Therefore, conventional methods for quantitation of helical content in CD spectra are not reliable for distinguishing small differences between two peptides.

Both of these methods for quantitation of the amount of helix formed were used to determine the helical content of all three peptides as a function of TFE content, and the results are shown in Table II. Both methods reveal significant differences in helical content between CPA and either MCPA1 or MCPA2 at high levels of TFE (>5%). However, quantitative results obtained on CPA and MCPA1 in the absence of TFE are within experimental error of each other. Furthermore, quantitative results for MCPA1 and MCPA2 are within experimental error at all levels of TFE. Since the qualitative shapes of the CD spectra are reproducibly different, these results suggest that the existing methods for quantitation of α-helical content are not sufficiently sensitive to measure accurately small differences.

The above discussion makes it clear that more sensitive and reproducible parameters are required for unambiguous comparison of the helix content of these three peptides. It is desirable for these parameters to be independent of peptide concentration (to eliminate error from concentration determination), and these parameters also must be sensitive measures of small changes in helical content, especially when the amount of helix is low. We propose two parameters, R1 and R2, which are ratios of intensities in CD spectra. Provided that the peptide conformation is independent of concentration (which is true for these peptides over a range of 5 to 30 μM), these two ratios are independent of the peptide concentration. The R1 and R2 parameters are based on the fact that in a two-state equilibrium between α-helix and random coil conformations, the absolute value of both $[\theta]_{222}$, the mean residue ellipticity at 222 nm, and $[\theta]_{max}$, the maximum ellipticity in the range from 190 to 195 nm, increase relative to $[\theta]_{min}$, the minimum ellipticity in the range from 195 to 210 nm, as the helical content of the peptide increases. Therefore, the ratio R1, defined as $[\theta]_{max}/[\theta]_{min}$, will be positive at low levels of helix formation ($[\theta]_{max}$ and $[\theta]_{min}$ both negative) and will steadily decrease to a limiting value near −2 ($[\theta]_{max}$ positive and $[\theta]_{min}$ negative) as the helical content of the peptide is increased. Alternatively, the ratio R2, defined as $[\theta]_{222}/[\theta]_{min}$, will be approximately zero in the absence of any helix formation and will steadily increase to a limiting value near 1 (or somewhat greater, as the $[\theta]_{222}$ minimum is sometimes deeper than that of 208 nm) as the amount of helix is increased. The ratios R1 and R2 are useful probes of the *relative* amount of α-helix present in two or more similar peptides. Since R1 and R2 are primarily characteristics of the shape of the CD spectra, quantitation of helical content using these parameters

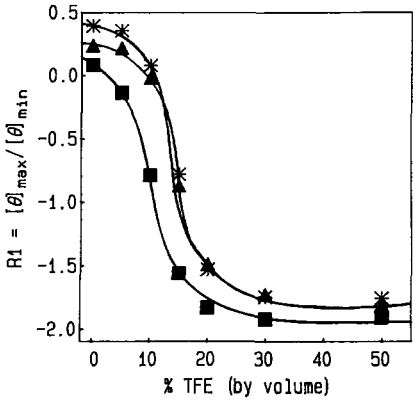

Fig. 3. Graph of R1 versus % TFE for the three peptides CPA (squares), MCPA1, (triangles), and MCPA2 (asterisks). The estimated uncertainty of R1 is ± 0.05; the diameter of the symbols is 0.10.

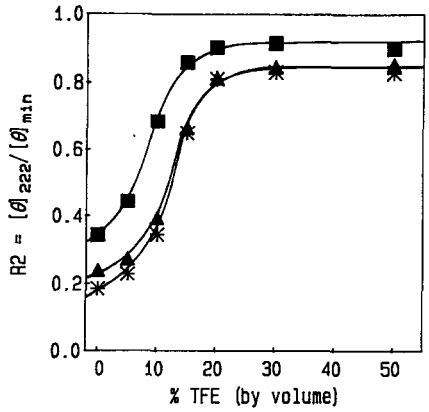

Fig. 4. Graph of R2 versus % TFE for the three peptides CPA (squares), MCPA1 (triangles), and MCPA2 (asterisks). The estimated uncertainty of R2 is ± 0.02; the diameter of the symbols is 0.04.

suffers from some of the same limitations as curve-fitting by shape of CD spectra to Greenfield-Fasman polypeptide reference spectra, as described earlier. However, use of R1 and R2 does not assume that the pure random coil and α-helical reference spectra of small peptides are the same as those observed for larger polymers; these parameters assume only that the reference spectra are essentially the same for similar peptides of the same length. It must be emphasized that these parameters are useful only for peptides whose conformations can be adequately described by a two-state α-helix/random coil equilibrium; the presence of β-structure would invalidate this approach.

Comparison of the helical content of the different peptides is facilitated by the titration curves of the ratios R1 (Fig. 3) and R2 (Fig. 4) as a function of TFE content. These curves are sigmoidal for all three peptides, and this is indicative of a cooperative transition from random coil to α-helix. The native peptide CPA has larger R2 values and more negative R1 values than the other two peptides for all levels of TFE, and this confirms the higher helical content of CPA. Not only does this peptide have more helix in the absence of TFE, it also can be induced to adopt a higher population of a helical conformation at high levels of TFE than the other two peptides. Furthermore, the midpoint of the random coil–helix transition of CPA is shifted to a lower TFE level (10%) relative to that observed for MCPA1 and MCPA2 (15%). These curves also show small, but significant, differences between MCPA1 and MCPA2 at low levels of TFE (<10%). However, both R1 and R2 are within experimental error for these two peptides at higher levels of TFE. The midpoint for the coil–helix transition is the same for the two peptides, and both peptides achieve nearly equal helical contents of 50% TFE.

The results described above demonstrate that CPA forms a more stable helix than the peptide MCPA1. Since MCPA1 differs from CPA by substitution of two neutral residues, Gln and Ser, for the two charged residues, Glu and His, in CPA, this difference is helical content is probably due to the influence of electrostatic interactions, most likely with the helix dipole, on helix stability (see below). The small differences observed between MCPA1 and MCPA2 in the absence of TFE must reflect the contribution of side chain–backbone hydrogen bonding of the NTB and CTB residues to helix stability. Interestingly, the effect of this hydrogen bonding is mitigated by the presence of an appreciable amount of TFE. It is difficult to determine the source of this mitigating effect of TFE because the exact mechanism by which TFE stabilizes helix formation is not known. Since TFE is a good hydrogen bond donor, formation of hydrogen bonds between the peptide and TFE may contribute to helix stability in a similar manner to side chain–backbone hydrogen bond formation. Therefore, TFE may obviate the necessity of NTBs and CTBs for hydrogen bonding interactions which contribute to helix stability. Alternatively, TFE may magnify the helix dipole effect by lowering the dielectric constant of the solvent and diminish the side chain–backbone hydrogen bonding effect by competing with sidechain acceptors in NTBs and backbone acceptors in CTBs.

pH Titrations

The results presented in the previous section suggest that helix formation in the peptide CPA is stabilized in part by electrostatic interactions; these interactions are not possible in the modified peptides MCPA1 and MCPA2. If electrostatic interactions are responsible for helix stability, the CD spectra of CPA should exhibit a dependence on the pH of the solution. The CD spectra of the three spectra obtained in buffer solutions of differing pH are shown

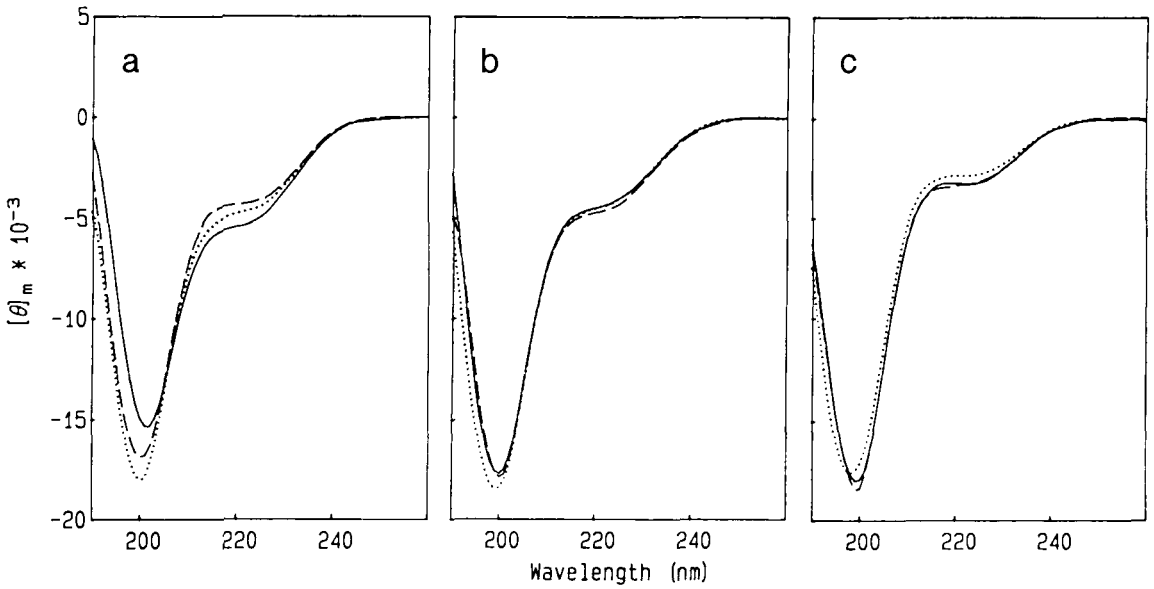

Fig. 5. CD spectra of (**a**) CPA, (**b**) MCPA1, and (**c**) MCPA2 in phosphate buffer at 25°C. The solid line represents pH 5.3, the dashed line pH 9.6, and the dotted line pH 2.6. For all peptides, the mean residue ellipticity is plotted (deg cm^2 $dmol^{-1}$).

in Figure 5. The spectra of CPA show qualitative differences indicative of reduced helix content at both low and high pH. This pH dependence suggests that electrostatic interactions between the charged groups, Glu-3 (p*K* of 4.25) and His-16 (p*K* of 6.0), and the helix dipole contribute significantly to helix stability for the native CPA peptide (as expected based on previous investigations into the sources of helix stability), although other specific interactions with these charged residues are also possible.[19] By contrast, less pH dependence is observed for the peptides MCPA1 (Fig. 1b) and MCPA2 (Fig. 1c). For these two peptides, the CD spectrum obtained at pH 5.3 is essentially indistinguishable from that obtained at pH 9.6. However, there is a small loss of helix at pH 2.6 for both peptides; this is surprising since the charged groups near the end of the CPA peptide have been removed in the modified peptides MCPA1 and MCPA2. Initially, we thought this sensitivity of the CD spectrum to low pH was due to interactions with Glu-5. To probe this hypothesis, a new peptide, containing a E5→Q point substitution relative to MCPA1, was analyzed. Since this peptide also showed a slight loss of helix at low pH (data not shown), electrostatic interactions with Glu-5 cannot be the source of this destabilization. Alternatively, this low pH dependence can be explained by disruption of salt bridge formation. Potential salt bridges between Lys and Asp (p*K* of 3.9) in all three peptides would be weakened by protonation of aspartic acid at pH 2.6, and this could result in loss of α-helix at low pH. The removal of the majority of the pH dependence of the native CPA peptide by substitution of the charged residues Glu and His confirms that these two residues are important for helix stability of CPA.

Difference spectroscopy is a sensitive method for detecting small changes in the distribution between helical and random conformations,[20] and this method was used to confirm the interpretation of the pH titrations described above. The CD spectrum obtained at pH 9.6 was subtracted from the CD spectrum obtained at pH 5.3 for each of the three peptides (Fig. 6). The difference spectrum for CPA has the characteristic shape indicative of an α-helix, and this argues that the helical content of CPA increases at pH 5.3.[2,†] By contrast, the difference spectra for the peptides MCPA1 and MCPA2 do not display shapes characteristic of an α-helix, and both spectra have very low intensities at all wavelengths. These results confirm that there is little or no pH dependence (over the range from 5.3 to 9.6) associated with the conformational distribution for the two peptides MCPA1 and MCPA2.

To determine whether or not electrostatic interactions are still important in the presence of TFE, the pH titrations were repeated in the presence of 10% TFE (Fig. 7). The results are essentially the same as observed in the absence of TFE (Fig. 5), but the destabilizing effects are more dramatic since, in 10% TFE, all three peptides have more helix which can potentially be lost. As observed in buffer, the native CPA peptide suffers a significant loss of helix when

†Note that the difference spectrum for a two-state helix–coil equilibrium should be a linear combination of the changes in both states.

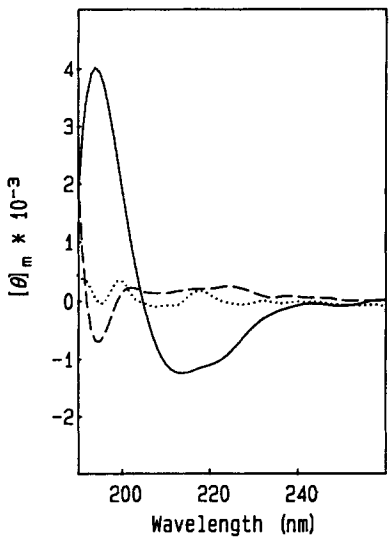

Fig. 6. Difference CD spectra for CPA (solid line), MCPA1 (dashed), and MCPA2 (dotted). For each peptide, the CD spectrum in pH 9.6 buffer was subtracted from the CD spectrum in pH 5.3 buffer.

the pH changes from 5.3 to 9.6 or 2.6. The other two peptides experience no loss of helix at high pH, but a small loss of helix is indicated at pH 2.6. These results imply that TFE does nothing to mitigate electrostatic interactions associated with charged residues in the peptide, including salt bridge formation as well as interactions with the helix dipole. Not surprisingly, these electrostatic interactions clearly provide a significant contribution to helix stability, especially in the native CPA peptide.

The qualitative results described above are supported by the ratios R1 and R2 measured as a function of pH. As observed for the TFE titration, the quantitative results obtained with conventional methods (Table III and IV) are not sufficiently sensitive to measure small, but highly reproducible, differences in the helical content of these peptides as a function of pH. However, these differences *are* reflected in the ratios R1 and R2 (Tables V and VI). The peptide CPA clearly has less helix at both low and high pH, whereas the only pH dependence observed for the peptides MCPA1 and MCPA2 is a small loss of helix at low pH.

It should be noted that the native peptide CPA has the potential to form an $i/i+4$ salt bridge between Glu-3 and Lys-7, and this potential salt bridge is removed in the modified peptides MCPA1 and MCPA2. Since the side chain pK values for Glu and Lys are 4.25 and 10.5, respectively, this potential salt bridge will be disrupted at pH 2.6, but not at pH 9.6, in the pH titration. In the peptide CPA, essentially the same amount of helix was lost at both high and low pH values relative to that observed at pH 5.3, and this demonstrates that the contributions to helix stability at pH 9.6 are not significantly greater that at pH 2.6. Therefore, this potential Glu-3$^-$ . . . Lys-7$^+$ salt bridge probably is not contributing significantly to helix stability in CPA.

CONCLUSIONS

We have analyzed the helix stability of three peptides (CPA, MCPA1, MCPA2) as a function of both pH and TFE. As anticipated based on previous studies, both pH and TFE titrations of the native CPA peptide indicate that the helix stability of this peptide is due in part to presence of the charged residues, Glu-3 and His-16, near the helix termini, most likely because of their interactions with the helix dipole. Furthermore, interactions with both ends of the helix dipole appear to be equally important for helix stability. However, differences observed between the helical contents of MCPA1 and MCPA2 demonstrate that side chain–backbone hydrogen bond formation also contributes to helix stability. This observation constitutes the first direct evidence in peptides in support of the helix hypothesis proposed by Presta and Rose.[1] In aqueous solution, the differences observed between CPA and MCPA1 are nearly equal in magnitude to the differences between MCPA1 and MCPA2. These observations suggest that, in the absence of TFE, hydrogen bonding and electrostatic interactions contribute nearly equally to helix stability. Contributions to helix stability from hydrogen bonding interactions are insignificant at high levels of TFE, which may reflect the hydrogen bonding capabilities of TFE itself.

TABLE III. pH Titration of CPA Peptides in Water: Estimation of % α-Helix

pH	CPA $[\theta]_{222}$*	% α-helix shape†	MCPAI $[\theta]_{222}$	% α-helix shape	MCPA2 $[\theta]_{222}$	% α-helix shape
2.6	15	25	14	20	9	15
4.2	17	25	14	25	11	20
5.3	17	30	14	25	11	20
6.8	14	25	14	25	11	20
9.6	14	25	15	25	11	20

*% α-helix determined from the intensity at 222 nm according to Eq. (1).[17] Estimated absolute error is ±1 for CPA, MCPA2 and ±2 for MCPA1.

†These values are obtained from curve fitting to polylysine reference spectra[18] and are rounded to the nearest 5%. Estimated absolute error is ±3.

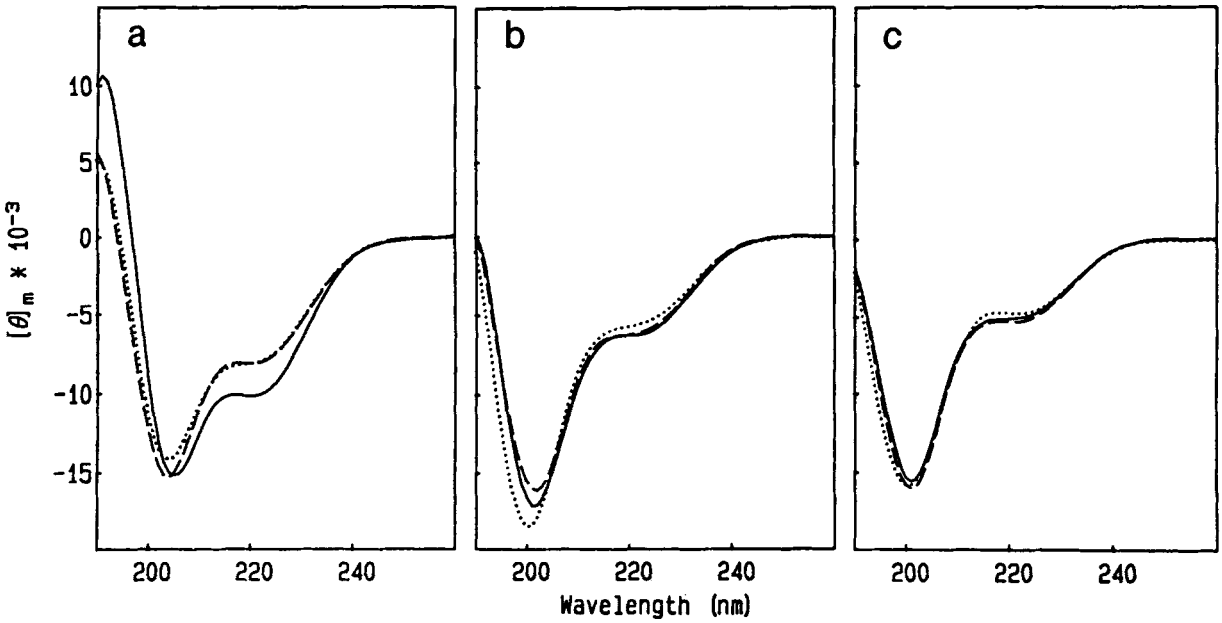

Fig. 7. CD spectra of (**a**) CPA, (**b**) MCPA1, and (**c**) MCPA2 in phosphate buffer containing 10% TFE (by volume) at 25°C. The solid line represents pH 5.3, the dashed line pH 9.6, and the dotted line pH 2.6.

TABLE IV. pH Titration of CPA Peptides in 10% TFE: Estimation of % α-Helix

pH	CPA $[\theta]_{222}$*	% α-helix shape†	MCPAI $[\theta]_{222}$	% α-helix shape	MCPA2 $[\theta]_{222}$	% α-helix shape
2.6	26	35	18	25	15	25
4.2	31	45	19	30	17	25
5.3	33	45	20	28	16	29
6.8	26	40	‡	‡	‡	‡
9.6	26	30	20	30	17	25

*% α-helix determined according to Eq. (1).[17] Estimated absolute error is ±3.
†These values are obtained from curve fitting to polylysine reference spectra.[18] Estimated absolute error is ±5.
‡Not measured.

TABLE V. pH Titration of CPA Peptides: The Ratio R1*

	Buffer only			10% TFE in buffer	
pH	CPA	MCPA1	MCPA2	CPA	MCPA1	MCPA2
2.6	+0.26	+0.28	+0.42	−0.37	+0.05	+0.13
4.2	+0.16	+0.27	+0.38	−0.68	+0.11	+0.09
5.3	+0.08	+0.24	+0.39	−0.79	−0.02	+0.08
6.8	+0.20	+0.18	+0.43	−0.49	†	†
9.6	+0.17	+0.28	+0.37	−0.37	−0.01	+0.13

*R1 is defined as the ratio of the maximum intensity from 190 to 195, $[\theta]_{max}$, to the minimum intensity in the range from 195 to 210 nm, $[\theta]_{min}$. The estimated absolute error is ±0.05.
†Not measured.

It must be remembered that, based on composition alone, the intrinsic helical propensity is predicted to be higher in MCPA2 than in MCPA1 due to the large number of alanines in the former peptide. This high intrinsic helix probability of MCPA2 may raise the helical content of this control peptide, which is lacking both hydrogen bonding side chains and charged residues. Another control peptide probably will have less helix than MCPA2, and this would make the difference between the peptides greater. Consequently, the contribution to helix stability due to the hydrogen bonding model is probably larger than is indicated by the difference between MCPA1 and MCPA2.

TABLE VI. pH Titration of CPA Peptides: The Ratio R2*

	Buffer only			10% TFE in buffer	
pH	CPA	MCPA1	MCPA2	CPA	MCPA1	MCPA2
2.6	0.26	0.22	0.16	0.56	0.30	0.30
4.2	0.32	0.23	0.18	0.65	0.36	0.34
5.3	0.34	0.24	0.19	0.69	0.39	0.34
6.8	0.26	0.26	0.19	0.55	†	†
9.6	0.25	0.26	0.18	0.53	0.38	0.33

*R2 is defined as the ratio of the intensity at 222 nm, $[\theta]_{222}$, to the minimum intensity in the range from 195 to 210 nm, $[\theta]_{min}$. The estimated absolute error is ±0.02.
†Not measured.

In this study, we also have introduced two new parameters, R1 and R2, which are useful for comparison of the helical content in different peptides. These parameters are independent of peptide concentration since they are ratios of intensities in the CD spectrum. We have demonstrated that differences in these ratios are sensitive probes of small changes in helical content, especially when only a small amount of helix is present. Therefore, these ratios provide a precise, concentration independent, basis for comparison of the amount of helix present in different peptides.

ACKNOWLEDGMENTS

We thank Candace Millhouse, Carolyn Moomaw, Khuan Ng, and Sarah Stradley for expert technical assistance with synthesis, purification, and analysis of peptides. This research has been supported in part by the National Institutes of Health (GM27616) and the Robert A. Welch Foundation.

REFERENCES

1. Presta, L.G., Rose, G.D. Helix signals in proteins. Science 240:1632–1641, 1988.
2. Bierzynski, A., Kim, P.S., Baldwin, R.L. A salt bridge stabilizes the helix formed by isolated C-peptide of RNase A. Proc. Natl. Acad. Sci. U.S.A. 79:2470–2474, 1982.
3. Shoemaker, K.R., Kim, P.S., Brems, D.N., Marqusee, S., York, E.J., Chaiken, J.M., Stewart, J.M., Baldwin, R.L. Nature of the charged-group effect on the stability of the C-peptide helix. Proc. Natl. Acad. Sci. U.S.A. 82:2349–2353, 1985.
4. Shoemaker, K.R., Kim, P.S., York, E.J., Stewart, J.M., Baldwin, R.L. Tests of the helix dipole model for stabilization of α-helices. Nature (London) 326:563–567, 1987.
5. Fairman, R., Shoemaker, K.R., York, E.J., Stewart, J.M., Baldwin, R.L. Further Studies of the helix dipole model: Effects of a free α-NH_3^+ or α-COO^- group on helix stability. Proteins: Struct. Funct. Genet. 5:1–7, 1989.
6. Marqusee, S., Baldwin, R.L. Helix stabilization by Glu . . . Lys^+ salt bridges in short peptides of *de novo* design. Proc. Natl. Acad. Sci. U.S.A. 84:8898–8902, 1987.
7. Lyn, P.C., Marky, L.A., Kallenbach, N.R. The role of ion pairs in α-helix stability: Two new designed helical peptides. J. Am. Chem. Soc. 111:2733–2734, 1989.
8. Serrano, L., Fersht, A.R. Capping and α-helix stability. Nature (London) 342:296–299, 1989.
9. Rees, D.C., Lewis, M., Lipscomb, W.N. Refined structure of carboxypeptidase A at 1.54 A resolution. J. Mol. Biol. 168: 367–387, 1983.
10. Chou, P.Y., Fasman, G.D., Prediction of protein conformation. Biochemistry 13:211–245, 1974.
11. Sueki, M., Lee, S., Powers, S.P., Denton, J.B., Konishi, Y., Scheraga, H.A. Helix-coil stability constants for the naturally occurring amino acids in water. 22. Histidine parameters from random poly[(hydroxybutyl)glutamine-CO-L-histidine]. Macromolecules 17:148–155, 1984.
12. Marqusee, S., Robbins, V.H., Baldwin, R.L. Unusually stable helix formation in short alanine-based peptides. Proc. Natl. Acad. Sci. U.S.A. 86:5286–5290, 1989.
13. S. Padmanabhan, Marqusee, S., Ridgeway, T., Lau, T.M., Baldwin, R.L. Relative helix-forming tendencies of nonpolar amino acids. Nature (London) 344:268–270, 1990.
14. Woody, R.W. The circular dichroism of peptides. In: "The Peptides. Vol. 7." Hruby, V.J., ed. New York: Academic Press, 1985: pp. 15–114.
15. Nelson, J.W., Kallenbach, N.R. Stabilization of the ribonuclease S-peptide α-helix by trifluoroethanol. Proteins: Struct. Funct. Genet. 1:211–217, 1988.
16. Nelson, J.W., Kallenbach, N.R. Persistence of the α-helix stop signal in the S-peptide in trifluoroethanol solution. Biochemistry 28:5256–5261, 1989.
17. Chen, Y.H., Yang, J.T., Chau, K.H. Determination of the helix and β forms of proteins in aqueous solution by circular dichroism. Biochemistry 13:3350–3359, 1974.
18. Greenfield, N., Fasman, G.D. Computed circular dichroism spectra for the evaluation of protein conformation. Biochemistry 8:4108–4115, 1969.
19. Shoemaker, K.R., Fairman, R., Schultz, D.A., Robertson, A.D., York, E.J., Stewart, J.M., Baldwin, R.L. Side-chain interactions in the c-peptide helix: Phe8 . . . $His12^+$. Biopolymers 29:1–11, 1990.
20. Goodman, E.M., Kim, P.S. Folding of a peptide corresponding to the α-helix in bovine pancreatic trypsin inhibitor. Biochemistry 28:4343–4347, 1989.