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Positional Independence and Additivity of Amino Acid Replacements on Helix Stability in Monomeric Peptides†

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ABSTRACT: The 17-residue peptide acetylAEAAAKEAAAKEAAAKAamide, described as an autonomous folding unit (Marqusee & Baldwin, 1987), has been used to examine the effect of amino acid replacements on helix stability. Alanine residues(s) at positions 4, 9, and 14 in the peptide sequence were replaced either singly or multiply by either serine or methionine residues with solid-phase peptide synthesis. The thermal dependence of the helix/coil transition of each peptide was observed by far-ultraviolet circular dichroism. Within experimental variation, all three single replacements exhibit a common thermal transition, and all three double replacements exhibit a different common thermal transition. These results suggest that replacement of the central alanine residue in the repeat EAAAK located in the N-terminus, in the middle, or in the C-terminus of the peptide helix has the same effect on helix stability. The melting temperature of each thermal transition was estimated by assuming a linear van't Hoff plot and a change in molar ellipticity of 33 500 deg cm^2 dmol^{-1}. Such analysis indicates that each replacement of an alanine residue by a serine residue diminishes the melting temperature by 11 ± 1 °C and that each replacement of an alanine residue by a methionine residue diminishes the melting temperature by 6 ± 1 °C. These results suggest that the effect of these replacements on helix stability is additive.

During the last several years, it has been demonstrated that analogues of the N-terminal peptide of pancreatic ribonuclease exhibit the spectral features of a monomeric helix/coil transition in aqueous solution (Shoemaker et al., 1985, 1987). It is observed that the helical content of the peptide can be enhanced significantly by optimization of the distribution of charged groups relative to the helix dipole. Recently, it has been reported that the monomeric helical form of the 17-residue peptide acetylAEAAAKEAAAKEAAAKAamide can be a major component in aqueous solution (Marqusee & Baldwin, 1987). This peptide provides a model system to examine the inherent propensity of amino acids to stabilize helicity unfettered by the packing considerations that occur in protein structures. We plan to generate systematic analogues of this parent peptide to examine the relationship between their helical content and the amino acid residue helix probabilities obtained from analysis of crystallographic structures. Since 20^{17} sequence analogues of this peptide exist,

Table I: Peptide Sequences

sequence	notation
acetylAEAAAKEAAAKEAAAKAamide	AAA
acetylAEASAKEAAAKEAAAKAamide	SAA
acetylAEAAAKEASAKEAAAKAamide	ASA
acetylAEAAAKEAAAKEASAKAamide	AAS
acetylAEASAKEASAKEAAAKAamide	SSA
acetylAEASAKEAAAKEASAKAamide	SAS
acetylAEAAAKEASAKEASAKAamide	ASS
acetylAEASAKEASAKEASAKAamide	SSS
acetylAEAMAKEAAAKEAAAKAamide	MAA
acetylAEAAAKEAMAKEAAAKAamide	AMA
acetylAEAAAKEAAAKEAMAKAamide	AAM
acetylAEAMAKEAMAKEAAAKAamide	MMA
acetylAEAMAKEAAAKEAMAKAamide	MAM
acetylAEAAAKEAMAKEAMAKAamide	AMM
acetylAEAMAKEAMAKEAMAKAamide	MMM

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it is crucial to reduce the scope of the study to a more manageable size. In this paper, we investigate (a) whether the helical content is sensitive to the location of a given replacement in a sequence and (b) whether the helical content exhibits an additive response to the numbers of the given replacement in the sequence. Inspection of residue side-chain structures and available helix probability values suggested that replacement of an alanine residue by a serine residue should diminish helical content without significantly perturbing dipolar or steric considerations. Similarly, replacement of an alanine residue by a methionine residue should enhance helical content without significant generation of such perturbations. The alanine residues targeted for replacement are located at positions 4, 9, and 14 in the parent peptide sequence. The sequences of the 15 peptides used in this study and their notation are listed in Table I. It should be noted that the replacements occupy the central position, in the pentapeptide repeat, EAAAK, within the parent peptide sequence. Thus, the position of the replacement is varied as to its location in the N-terminal, in the middle, or in the C-terminal portion of the helix without alteration of its near-neighbor environment. We find that helix stability is independent of the position of a given replacement and directly dependent upon the number of such replacements.

Materials and Methods

Materials. *p*-Methylbenzhydrylamine resin (150–200 mesh, 0.7 mequiv/g) was purchased from Colorado Biotechnology Assoc., Boulder, CO. *t*-Boc-amino acids containing the most common side-chain blocking groups were purchased from Bachem Inc., Torrance, CA. HPLC-grade acetonitrile, dichloromethane, dimethylformamide, and 2-propanol were obtained from Burdick and Jackson, Muskegon, MI. Biograde trifluoroacetic acid was purchased from Halocarbon Laboratories, North Augusta, SC. Fluorinert FC-77 was obtained from 3M, St. Paul, MN. Polypropylene mesh (74 μm) was purchased from Spectrum Medical Industries, Inc., Los Angeles, CA. All other peptide synthesis chemicals were obtained from Aldrich Chemical Co., Milwaukee, WI. All other reagents were reagent grade or better.

Peptide Synthesis. Peptides used in this paper were synthesized with the simultaneous multiple-peptide synthesis method (Houghten, 1985; Houghten et al., 1986). One hundred milligrams of *p*-methylbenzhydrylamine resin was placed in each of a series of 35 × 35 mm polypropylene bags constructed with an impulse sealer. Each bag was labeled, closed, washed twice with dichloromethane, and weighed to check for loss of resin. All bags were placed in a wide-mouth polypropylene bottle preparatory to coupling the C-terminal residue common to all the peptides. Coupling was carried out in a solution of 0.2 M *t*-Boc-amino acid and 0.2 M diisopropylcarbodiimide dissolved in dichloromethane in a final volume of 4 mL/bag. The coupling mixture was shaken for 1 h at ambient temperature with a Wheaton shaker table at 350 rpm. After the coupling reaction, bags were washed sequentially with dimethylformamide and dichloromethane. The bags were transferred to a common container, and the peptide amino terminus was deprotected by reaction with a solution of 55% trifluoroacetic acid, 43% dichloromethane, and 2% *p*-cresol for 30 min. The bags were then washed sequentially with dichloromethane, 2-propanol, and dichloromethane containing 5% diisopropylethylamine prior to coupling the next residue. After addition of the last amino acid, the N-terminus of the peptide was acetylated by reaction with 10% acetic anhydride, 10% diisopropylethylamine, and 80% dimethylformamide for 1 h. The bags were then washed with dichloromethane, air-dried, and weighed to estimate the amount of peptide synthesized. The peptide side-chain protecting groups were removed, and the peptide was cleaved from the resin by reacting each bag with 25 mL of a mixture of 90% HF and 10% *p*-cresol at 0 °C for 1 h. After 1 h, excess HF was removed under vacuum at 0 °C. Each bag was extracted with ethyl ether and then with 15% acetic acid. In most cases, the majority of the desired peptide appeared as a whitish precipitate in the ether extract. This precipitate was dissolved in a small volume of water, separated from the ether, and lyophilized.

Peptide Fractionation. Each peptide preparation was purified by reversed-phase chromatography using a 7 × 300 mm Hamilton PRP-1 column and an IBM LC/9533 chromatograph. About 20 mg of peptide was injected and partitioned in a linear gradient between 5% and 40% acetonitrile in 0.1% trifluoroacetic acid which was generated in 50 min at a flow rate of 3 mL/min at ambient temperature. The column effluent was monitored at 220 nm with an Isco Model V4 absorbance detector and collected in 1.5-mL fractions. Fractions containing the major component in the column effluent were pooled, lyophilized, and dissolved in water. These solutions were used as the source material for all further experimentation. A 10-μL aliquot of each peptide solution was subjected to analytical reversed-phase chromatography using a 4.6 × 100 mm C18 Spherisorb ODS2 column containing 3-μm particles and 120000 plates/m. Samples were partitioned in a linear gradient between 10% and 50% acetonitrile in 0.1% trifluoroacetic acid which was generated in 15 min at a flow rate of 1 mL/min at ambient temperature. The resolution of this protocol was investigated with the 17-residue peptide having amino acid replacements at position 9. Replacements that have a net effect of addition of a methyl group (S/T and V/I) change the retention time by 0.5–0.9 min, addition of a methylene group (D/E and N/Q) by 0.2–0.5 min, the addition of an oxygen (F/Y) by 1.2 min. All peptide solutions used in the study reported here were found to have a major component that comprised at least 90% of the absorbing material. The apparent purity of representative peptides AAM, AAS, and AMM was not diminished by slowing the gradient generation to 40 min. The elution position of the major component in each chromatogram exhibited a systematic dependence on the abundance of serine and methionine. This dependence presumably reflects the relative hydrophobicity of serine, alanine, and methionine.

The amino acid composition of each peptide was determined on a Waters Pico-Tag analyzer following gas-phase hydrolysis in HCl for 24 h at 110 °C. These analyses were performed by the staff of the Protein Structure Facility at the University of Iowa. The lysine/glutamate compositional ratio was 1.00 ± 0.06 for all of the peptides. The alanine/lysine ratios were 3.24 ± 0.21, 3.03 ± 0.18, and 2.65 ± 0.06 for peptides containing one, two, and three substitutions, respectively. The alanine/serine ratios were 9.52 ± 0.31, 4.41 ± 0.07, and 2.70 for peptide containing one, two, and three serine residues, respectively. The alanine/methionine sulfone ratios were 9.24 ± 0.51, 4.41 ± 0.21, and 2.48 for peptides containing one, two, and three methionine residues, respectively. Methionine was oxidized to methionine sulfone by performic acid oxidation prior to acid hydrolysis.

Peptides AAM, AMM, and SSS were chosen at random for mass spectral analysis. These analyses were performed by the staff at the University of Iowa Mass Spectrometry Facility using a VG analytical ZAB-HF instrument, xenon gas, a matrix consisting of a 4:1 mixture of dithioerythritol

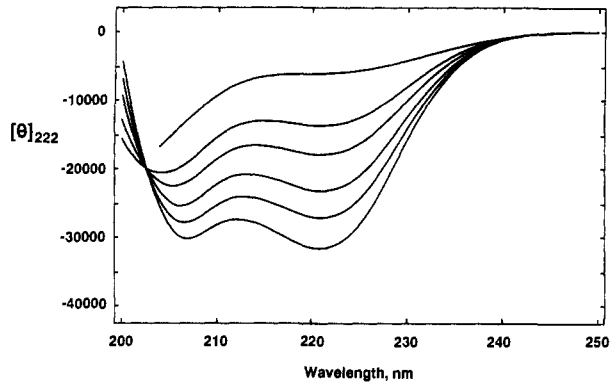

FIGURE 1: Thermal dependence of the far-ultraviolet circular dichroic spectrum of AAA on temperature. All spectra were obtained from a solution containing 30.4 μM peptide in 10 mM NaCl and 1 mM phosphate buffer, pH 7.0, placed in a stirred stoppered cuvet having an optical path of 10 mm. Individual spectra were recorded after the ellipticity at each temperature had attained a constant value at 222 nm. The spectra at 220 nm were obtained at 1, 16, 26, 36, 56, and 85 °C, reading upward. Spectra obtained above 60 °C could not be observed below 205 nm owing to the high absorbance of the solvent.

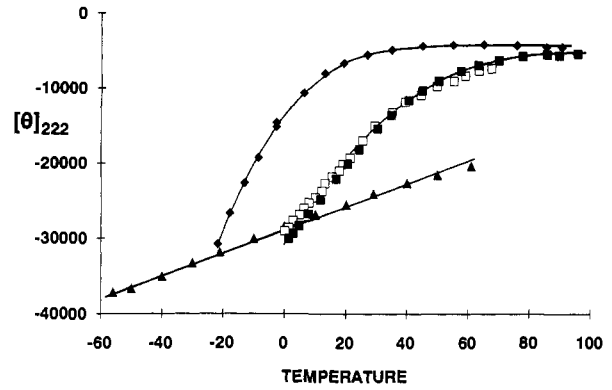

FIGURE 2: Thermal dependence of the residue molar ellipticity of AAA in various solvents. The filled and open squares represent values obtained in 10 mM NaCl and 1 mM phosphate buffer, pH 7. The filled squares represent values obtained in this study, and the open squares represent values reported by Marqusee and Baldwin (1987). The diamonds represent values obtained in 5 M NaCl and 1 mM phosphate buffer, pH 7. The triangles represent values obtained in 85 mol % trifluoroethanol.

and dithiothreitol, and the equivalency of 8 kV at a current of 1.5 mA. The major component in the spectra for peptides AAM and SSS was within 1 mass unit of that expected for the assumed peptide sequence. The major component in the spectrum for peptide AMM was within 1 mass unit of that anticipated for a fragment containing the first 11 residues of the acetylated peptide. The second most abundant component in this sample was within 1 mass unit of that anticipated for the unfragmented peptide.

Spectral Measurements. Circular dichroic measurements were obtained on an Aviv Associates Model 60DS spectropolarimeter located in the University of Iowa Protein Structure Facility. This instrument was calibrated with solutions of *d*-10-camphorsulfonic acid and *d*-pantolactone having molar ellipticities of 7780 deg cm^2 dmol^{-1} at 290.5 nm (Johnson, 1985) and −13 500 deg cm^2 dmol^{-1} at 221 nm (Konno et al., 1975), respectively. Optical cells having a path length of either 1 or 10 mm were placed in a thermostated cell holder and brought to the desired temperature by circulation of water from a Neslab Model RTE-4DD refrigerated bath or by circulation of Fluorinert FC-77 from a FTS Systems Model RC-200-80 recirculating cooler. The dichroic spectrum was recorded after the ellipticity at 222 nm was observed to attain a constant value at each temperature reported. All observed dichroic spectra were corrected for the ellipticity of the solvent and adjusted when necessary to ellipticity values of zero in the wavelength range 250–280 nm. Observed ellipticity values are expressed as molar residue ellipticity, $[\theta]$, with units of deg cm^2 dmol^{-1}, from peptide concentrations determined by amino acid analysis. This concentration was calculated from the assumed sequence and the alanine content of an aliquot from each peptide stock solution.

Results and Discussion

The effect of temperature on the far-ultraviolet circular dichroic spectrum of representative peptide AAA in 10 mM NaCl and 1 mM phosphate buffer, pH 7.0, is illustrated in Figure 1. At low temperatures the dichroic spectrum exhibits spectra having negative maxima at 207 and 221 nm, characteristic for the presence of a significant population of peptide bonds in a helical conformation (Holzwarth & Doty, 1965). As the temperature of the solution is increased, the bimodal spectrum is gradually transformed into a relatively featureless dichroic spectrum that persists at elevated temperatures and that is characteristic for denatured proteins at ambient temperatures (Cortijo et al., 1973; Rossi et al., 1983; Rudolph et al., 1986). The occurrence of an isosbestic point at 203 nm suggests that the spectral changes reflect a two-state transition between the helical and coil forms of the peptide bonds and that the coil form predominates at elevated temperatures. In this presentation, we have assumed that all the residues in a peptide are in a helical conformation and that each peptide in such a helix exhibits the same thermal transition.

The dependence of the molar ellipticity of peptide AAA at 222 nm on temperature in 10 mM NaCl and 1 mM phosphate buffer is denoted by the filled squares in Figure 2. The dependence corresponds with that previously reported (Marqusee & Baldwin, 1987) and presumably represents the upper portion of a sigmoidal transition. In an effort to demonstrate the entire sigmoidal transition, dichroic measurements were made in solvents that remain liquid below 0 °C. While the presence of 5 M NaCl in the peptide solvent facilitates measurements at lower temperatures, it also appears to destabilize the helix. The net effect of these two features does not increase the span of the observed transition as illustrated by Figure 2. The presence of 85 mol % trifluoroethanol in the peptide solvent facilitates measurements to at least −60 °C. Unfortunately, the presence of this solvent also diminishes the cooperativity of the transition such that only the central nearly linear portion is observed in the temperature range, −60 to +60 °C, in which this solvent is liquid. On the basis of these experiences, we have chosen to observe the helix/coil transition of the remaining peptides in 10 mM NaCl and 1 mM phosphate buffer, pH 7, because this solvent is more typical of those commonly used and because the alternative solvents offer no further analytical advantages.

The observed ellipticity at 222 nm of three representative peptides, AAA, MMA, and ASS, is linearly dependent upon peptide concentration up to a concentration of 400 μM. This concentration is at least an order of magnitude greater than the concentration used, 12–33 μM, to characterize the thermal transition of the 15 peptides. Accordingly, it is likely that the observed differences in the thermal transitions among the 15 peptides do not result from aggregation of either the helical or coil forms of the peptides.

All 15 peptides displayed a mean limiting molar ellipticity at 222 nm at elevated temperatures of −6500 deg cm^2 dmol^{-1} with a standard deviation of ±1200 deg cm^2 dmol^{-1}. To aid the visual comparison of the thermal transitions observed for

Table II: Dichroic Transitions

peptide	T_m (°C)	ΔT_m	$(\Delta T_m/\text{Ser})_{av}$	peptide	T_m (°C)	ΔT_m	$(\Delta T_m/\text{Met})_{av}$
AAA	22	0		AAA	22	0	
SAA	11	−11		MAA	17	−5	
ASA	9	−13	−11	AMA	15	−7	−6
AAS	14	−8		AAM	17	−5	
SSA	−1	−23		MMA	7	−15	
SAS	2	−20	−11	MAM	10	−12	−7
ASS	1	−21		AMM	9	−13	
SSS	−7	−29	−10	MMM	0	−22	−7

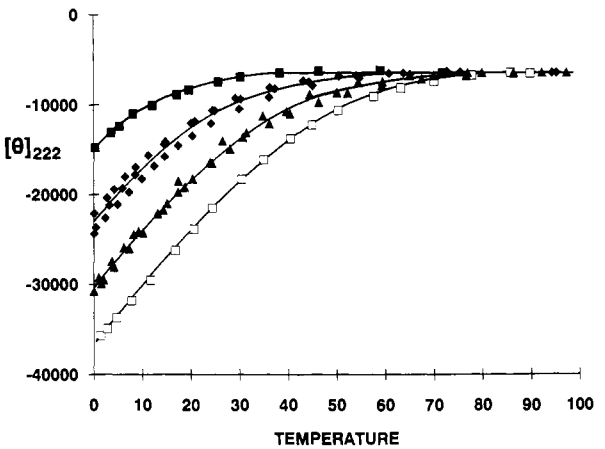

FIGURE 3: Thermal transitions for serine-containing peptides. Each observed transition was multiplied by a constant so that its ellipticity at elevated temperature had a value of −6500 deg cm^2 dmol^{-1}. The filled triangles represent values for each of the three peptides containing a single serine residue, SAA, ASA, and AAS. The filled diamonds represent values for each of the three peptides containing two serine residues, SSA, SAS, and ASS. The filled squares represent values for the peptide containing three serine residues, SSS. The open squares represent values for the reference peptide, AAA. All measurements were obtained at 222 nm in 10 mM NaCl and 1 mM phosphate buffer, pH 7.

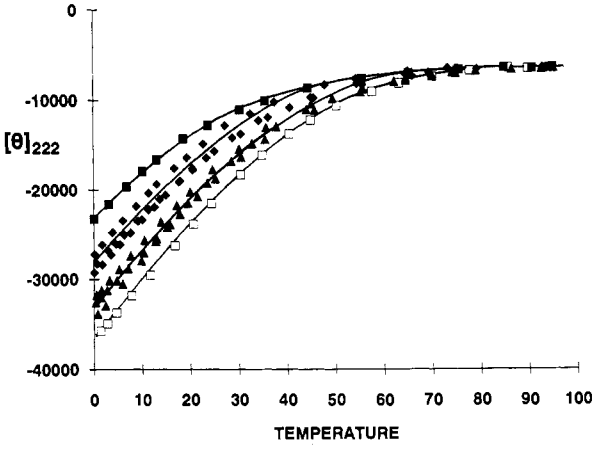

FIGURE 4: Thermal transitions for methionine-containing peptides. Each observed transition was multiplied by a constant so that its ellipticity at elevated temperature had a value of −6500 deg cm^2 dmol^{-1}. The filled triangles represent values for each of the three peptides containing a single methionine residue, MAA, AMA, and AAM. The filled diamonds represent values for each of the three peptides containing two methionine residues, AMM, MAM, and MMA. The filled squares represent values for the peptide containing three methionine residues, MMM. The open squares represent values for the reference peptide, AAA. All measurements were obtained at 222 nm in 10 mM NaCl and 1 mM phosphate buffer, pH 7.

the peptides, we have normalized the observed thermal transition of each peptide to the mean limiting molar ellipticity at elevated temperature. As shown in Figure 3, the thermal transition for the serine-containing peptides falls into three groups having one, two, or three serine residues. The same observation appears to pertain to the methionine-containing peptides even though the spacing between groups is less pronounced as shown in Figure 4. These comparisons suggest that the contribution of a residue to helix stability is to a first approximation independent of its location in the helix.

In order to examine the additivity of the contribution of a given residue to helix stability, we needed an estimate for the limiting ellipticity of the completely helical conformation of each peptide. Since the coil forms of all 15 peptides exhibit a mean molar ellipticity value with a modest deviation, we assume the same pertains to the helical form of each of the 15 peptides. We further assume that the central portion of the thermal transition of each peptide would display a linear van't Hoff plot. A computer program was then written to identify the change in the molar ellipticity between the helix and coil forms which would generate the smallest χ^2 deviation of the observed ellipticity values for each of the peptides from a linear van't Hoff plot. A blind test of the program demonstrated that the correct change in ellipticity was predicted if at least the upper third of the thermal transition was observed. Analysis of the normalized thermal transitions for the 15 peptides gave a common change in ellipticity of 33 500 deg cm^2 dmol^{-1}. The ellipticity of the helical form of all the peptides would then be −40 000 deg cm^2 dmol^{-1}, a value that is within the range of values, −36 000 to −40 000 deg cm^2 dmol^{-1}, obtained by either conventional analysis of homopeptides (Holzwarth & Doty, 1965; Greenfield & Fasman, 1969; Fillipi et al., 1978; Toniolo et al., 1979) by deconvolution of high-resolution crystallographic structures (Chang et al.,

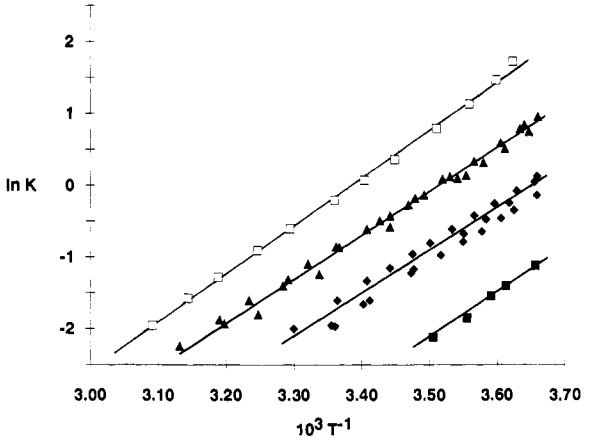

FIGURE 5: van't Hoff plots for the serine-containing peptides. The equilibrium constant K = [helix]/[coil] = $([\theta]_{obs} - [\theta]_{coil})/([\theta]_{helix} - [\theta]_{obs})$, where $[\theta]_{coil} = [\theta]_{obs}$ at the high-temperature plateau and $[\theta]_{helix} = [\theta]_{coil} - 33\,500$. The open squares represent values for the peptide containing no serine residues; the filled triangles represent values for the three peptides which each contain one serine residue; the filled diamonds represent values for the three peptides which each contain two serine residues, and the filled squares represent values for the peptide containing three serine residues. The individual lines were obtained by least-squares analyses of the experimental values. All measurements were obtained in 10 mM NaCl containing 1 mM phosphate buffer, pH 7.

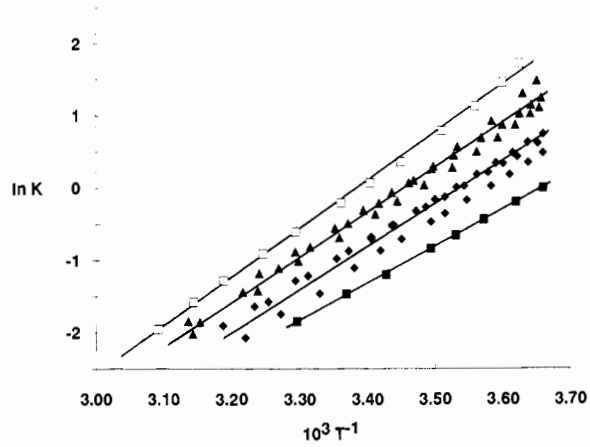

FIGURE 6: van't Hoff plots for the methionine-containing peptides. The equilibrium constant K = [helix]/[coil] = $([\theta]_{obs} - [\theta]_{coil})/([\theta]_{helix} - [\theta]_{obs})$, where $[\theta]_{coil} = [\theta]_{obs}$ at the high-temperature plateau and $[\theta]_{helix} = [\theta]_{coil} - 33\,500$. The open squares represent values for the peptide containing no methionine residues; the filled triangles represent values for the three peptides which each contain one methionine residue; the filled diamonds represent values for the three peptides which each contain two methionine residues, and the filled squares represent values for the peptide containing three methionine residues. The individual lines were obtained by least-squares analyses of the experimental values. All measurements were obtained in 10 mM NaCl containing 1 mM phosphate buffer, pH 7.

1978). From this change in ellipticity to define the molar ellipticity of the helical form of each peptide, the resultant van't Hoff plots for the 15 peptides are shown in Figures 5 and 6. The slopes of these plots have a mean value of 5940 K with a standard deviation of ±460 K. The mean value corresponds to an enthalpy change of 3.0 kcal/mol for the helix/coil transition of the 15 peptides. The melting temperature for each peptide is listed in Table II. On the basis of this analysis, it would appear that the stability of the peptide helix is diminished by 11 ± 1 °C for each serine residue in the peptide and by 6 ± 1 °C for each methionine residue in the peptide.

The positional independence and the additivity evident in the contributions of these replacements on helix stability would afford a relatively simple algorithm for helix prediction based on sequence analysis. The positional independence of the replacements contrasts with analyses of crystallographic structures (Argos & Palau, 1982) which predict that the helical propensity of serine can vary from 0.69 in the middle of a helix to 1.01 in the N-terminus of the helix and that the helical propensity of methionine can vary from 0.91 in the N-terminus to 1.66 in the middle. Further, the nonpositional helical propensities (Levitt, 1978) predict that replacement of alanine, propensity 1.29, by methionine, propensity 1.47, should enhance helix stability while it is observed to diminish peptide helix stability. These differences between residue helix propensities based on crystallographic analyses and on peptide characterization may reflect the contribution of helix packing to the propensity values derived from the analysis of crystallographic structures.

ACKNOWLEDGMENTS

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Registry No. AAA, 113852-19-0; SAA, 124175-19-5; ASA, 124175-20-8; AAS, 124175-21-9; SSA, 124175-22-0; SAS, 124175-23-1; ASS, 124175-24-2; SSS, 124175-25-3; MAA, 124175-26-4; AMA, 124175-27-5; AAM, 124175-28-6; MMA, 124175-29-7; MAM, 124199-82-2; AMM, 124175-30-0; MMM, 124175-31-1.

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