Stabilization of Helical Domains in Short Peptides Using Hydrophobic Interactions†

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ABSTRACT: The contribution of hydrophobic interactions in the stabilization of helical structure was compared for a series of short peptides that incorporated two ϵ-(3,5-dinitrobenzoyl)Lys residues at various positions. Results showed that in aqueous/organic mixtures, methanol induced helical stability over a wider range and at higher concentration than trifluoroethanol (TFE); a similar degree of stability was seen in low mole fraction mixtures of TFE in water. Solvent mixture titrations in TFE/water demonstrated that helical stability was highest for the peptide having a pair of modified residues spaced by three other residues. Solvent mixture titrations in TFE/water appear to be useful in indicating the degree of hydrophobic stabilization.

The factors that modulate peptide/protein conformations and interactions can be considered among the most basic elements of biochemistry. Despite intense investigation, the relative contributions of electrostatic, hydrogen bonding, and hydrophobic forces are not well understood. Toward our goal in the selective recognition of solvent exposed functional groups in peptides and proteins, we have attempted to develop short peptides (14−20 amino acids) with strongly stabilized helical structure. Structural studies of such short peptides frequently use organic solvents (often trifluoroethanol) and low temperatures (0−5 °C) to increase helical stability. The helix stabilizing effect of trifluoroethanol (TFE)[1] is incompletely understood, but a primary factor must be the stabilization of the network of amide backbone hydrogen bonds in this solvent, which is less polar, and is a weaker hydrogen bond acceptor than water.

Interest in increasing the reliability of protein structural prediction has focused attention on comparisons of the helix-forming potential of each amino acid (Chakrabartty & Baldwin, 1993; Chakrabartty et al., 1991; Gans et al., 1991; Kemp et al., 1991; Lyu et al., 1990; Merutka et al., 1990; Merutka & Stellwagen, 1990; O'Neil & DeGrado, 1990; Padmanabhan et al., 1990; Wojcik et al., 1990) and in exploring other mechanisms to stabilize helical domains. The results from many investigations have indicated that helical stability can be increased by reducing the helix dipole moment [via capping (Chakrabartty et al., 1993a; Richardson & Richardson, 1988; Shoemaker et al., 1985, 1987; Strehlow & Baldwin, 1989) or interactions between side chain residues (Armstrong & Baldwin, 1993; Forood et al., 1993; Lockhart & Kim, 1993; Lyu et al., 1992)], salt bridge formation (Bierzynski et al., 1982; Marqusee & Baldwin, 1987; Merutka & Stellwagen, 1991), and the high frequency occurrence of alanine (Marqusee et al., 1989).

Although hydrophobic forces are known to be important in the stabilization of proteins, the magnitude of the effect has been the subject of some controversy. Residue replacement studies in proteins have indicated that the hydrophobic sequestering of each methyl or methylene unit contributes ~1.3 kcal/mol to protein stability (Pace, 1992; Privalov & Makhatadze, 1993; Rose & Wolfenden, 1993). Also, π/π interactions between the side chains of aromatic amino acids [frequently found in proximal pairs (Serrano et al., 1991)] contribute 1−2 kcal/mol (Hunter et al., 1991). These interactions are similar to, or greater than, the strength of hydrogen bonding or electrostatic interactions in water (Fersht, 1987).

Because of their role in protein stabilization, hydrophobic interactions are of great interest in the study of peptide model systems. In such peptide models, however, the addition of organic solvents, which favor moderately stable helical domains by increasing the strength of hydrogen bonding, will have a complex effect due to the concomitant reduction in the strength of hydrophobic interactions. Although there have been extensive studies on the helix-forming propensities of the amino acids, the effects of nonelectrostatic interactions (Shoemaker et al., 1985) have not received as much attention in model systems. This report describes the use of interactions between a pair of highly hydrophobic modified amino acids to stabilize helical structure of a 17 amino acid peptide in mixtures of TFE/water and methanol/water. These "hydrophobic" peptides show the unusual property of increasing structure at higher concentrations of water in TFE. At very high water concentration, however, the effect of hydrogen bond disruption dominates over the effect of nonelectrostatic interactions (hydrophobic, π/π, etc.), and the structural stability decreases.

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[1] Abbreviations: Standard three-letter abbreviations are used for all natural amino acids. All amino acids and their derivatives are of the natural, L-configuration. Side chain protecting groups or other derivatization are indicated within parentheses [e.g., Fmoc-Ser(tBu)]. Standard notations (e.g., α, β, γ, etc.) are used for NMR resonance assignments. AAA, amino acid analysis; Ac, acetyl; Aly, ϵ-acetyllysine; Boc, *tert*-butoxycarbonyl; BOP, benzotriazol-1-yloxytris(dimethylamino)phosphonium hexafluorophosphate; CD, circular dichroism; DCC, 1,3-dicyclohexylcarbodiimide; DCM, dichloromethane; DMF, dimethylformamide; DMSO, dimethylsulfoxide; Dnb, ϵ-(3,5-dinitrobenzoyl)-lysine; Et_2O, diethyl ether; EtOAc, ethyl acetate; Fmoc, 9-fluorenylmethoxycarbonyl; HOBt, 1-hydroxybenzotriazole hydrate; HPLC, high pressure liquid chromatography; MeOH, methanol; MNBA, *meta*-nitrobenzyl alcohol; NMR, nuclear magnetic resonance; Pfp, pentafluorophenol; TFA, trifluoroacetic acid; TFE, trifluoroethanol; THF, tetrahydrofuran; UV, ultraviolet.

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There have been several strategies employed to generate highly helical, monomeric short peptides, including the linkage of helical turns through metal ligation (Ghadiri & Choi, 1990; Ruan et al., 1990), disulfide bond formation (Jackson et al., 1991), side chain lactam bridges (Öspay & Taylor, 1992), and the use of end-terminal covalent templates (Kemp et al., 1991; Müller et al., 1993). As part of a study aimed toward the photochemical cross-linking of helical peptides, we prepared a number of sequences that incorporated hydrophobic derivatives of lysine. We were surprised to find a high degree of intrinsic helical stability in these systems and decided to investigate the origin of this phenomenon further.

The work described here focuses on comparisons of helical stability arising from hydrophobic interactions as a function of water concentration in aqueous TFE and methanol. To simplify this discussion, stabilizing interactions are separated into two types: electrostatic (encompassing hydrogen bonding, dipole, and salt bridge interactions) and hydrophobic (including van der Waals, π/π stacking, and water solvation effects). In some instances, however, π/π stacking interactions will be considered separately from other hydrophobic effects.

MATERIALS AND METHODS

Peptide Synthesis and Instrumentation. All chemicals were obtained from the Aldrich Chemical Company except where noted. Peptides were synthesized as C-terminal amides using Fmoc-PAL-PEG resin (Millipore) (0.75 g, 0.14 mequiv/g, 0.105 mmol) using a Millipore 9050 peptide synthesizer and conventional Fmoc solid-phase chemistry. Four equivalents of each *N*-protected amino acid (Millipore) were used with BOP/HOBt activation and a 1 h coupling time. Lysine and glutamic acid side chains were protected as their Boc- or *tert*-butyl ester derivatives, respectively. Peptides were acetylated by treatment of the resin with a solution of acetic anhydride (0.5 M) and pyridine (0.5 M) in DMF for 1 h. Peptide cleavage and deprotection were effected by treatment with a solution of trifluoroacetic acid (TFA) (9.0 mL), thioanisole (0.5 mL), ethanedithiol (0.3 mL), and anisole (0.2 mL) for 2 h. After precipitation and washing with Et_2O/hexanes (1:1), peptides were purified using a Waters 600E semipreparative HPLC controller with a 490E detector, and a 25 × 10 cm Delta-Pak C18 300 Å cartridge column inside a Waters radial compression module. An eluent gradient of 5−70% CH_3CN in water (with 0.1% TFA) over 40 min was used to afford 50−90 mg of purified (>95%) peptide. Purity was determined by analytical HPLC which was performed using a Rainin HP controller and a Rainin UV-C detector with a Rainin 250 × 4.6 mm 5 μm Microsorb C18 column and a gradient of 10−90% CH_3CN in water (with 0.1% TFA) over 20 min. Peptide FAB mass spectra were determined by the Midwest Center for Mass Spectroscopy [with partial support by the National Science Foundation, Biology Division (Grant No. DIR 9017262)]; other mass spectra were determined at the Department of Chemistry, University of Pittsburgh. Quantitative amino acid analysis and concentration determination were carried out at the University of Pittsburgh Protein Sequencing Facility using a Beckmann 6300 amino acid analyzer, with ninhydrin detection. Samples were prepared by a 24 h hydrolysis of the evacuated sample in 6 M HCl with 0.5% phenol at 110 °C. Nuclear magnetic resonance spectra were acquired using Bruker AM-300 and AM-500 spectrometers. Where indicated, ^{13}C NMR multiplicities were determined from DEPT 135° spectra (Morris, 1984) and are indicated as C, CH, CH_2, and CH_3. All analytical data were consistent with the desired sequence. Melting points (mp) were determined using an Electrothermal capillary melting point apparatus and are uncorrected. Molecular modeling studies were carried out using a Silicon Graphics 4D-35 workstation, with Macromodel (Mohamadi et al., 1990) and Sybyl (Tripos Associates, 1699 S. Hanley Rd., Suite 303, St. Louis, MO 63144-29130) software.

Circular Dichroism Measurements. Peptide concentrations were determined by quantitative amino acid analysis. Concentrations for Dnb peptides were subsequently determined by UV spectroscopy (in 6 M guanidinium chloride, 100 mM phosphate, pH 7.00) with $\epsilon_{240} = 5.97 \times 10^4$ M^{-1} cm^{-1}. CD spectra were determined using a Jasco 710 circular dichroism spectropolarimeter, which was calibrated using D-(−)-pantoyllactone (ellipticity = 19.0 mdeg at 0.15 mg/mL in water, 219 nm, 0.1 cm path). A water-jacketed cylindrical cell (0.1 cm, Helma) was used with a Neslab programmable thermal bath. HPLC grade water was obtained from Baxter Scientific and was buffered with 1.0 mM phosphate and 10 mM NaCl at pH 7.00. TFE was fractionally distilled from charcoal. Peptides were stored as lyophilized powders at −20 °C. Solvent mixture titration experiments were prepared using aliquots of ~500 μM peptide stock using Hamilton gas-tight syringes for a final concentration of ~50 μM (determined spectrophotometrically). For solvent mixtures in the low range of water concentration, a second peptide stock solution was prepared by lyophilizing a known amount of peptide and redissolving in TFE. In each titration, intermediate water concentration mixtures were duplicated at least four times using both peptide stock solutions, and the results were scaled to account for small (<5%) deviations between the two stock concentrations. The degree of helicity was determined by monitoring the ellipticity at 222 nm using the average of 301 data points collected over 60 s. Ellipticity is reported as mean residue ellipticity using $\Theta_{222} = (100\Theta_{obs})/(LNC)$, where Θ_{obs} is the measured ellipticity (mdeg), L is the path length (0.1 cm), N is the number of amino acids in the peptide (17), and C is the peptide concentration (in millimolar).

Synthesis of ε-(3,5-Dinitrobenzoyl)-Fmoc-Lys (Fmoc-Dnb). To a solution of 3,5-dinitrobenzoic acid (0.48 g, 2.26 mmol) and HOBt (0.31 g, 2.29 mmol) in DCM (20 mL) and DMF (1 mL) was added DCC (0.46 g, 2.22 mmol) at 0 °C, and after 40 min the precipitate was removed by filtratration. A slurry of Fmoc-Lys (Bachem CA) (0.75 g, 2.03 mmol) in DMSO (5 mL) was added to this solution, and the clarified mixture was stirred for 6 h. The solution was concentrated under reduced pressure to a yellow-orange solution. Water (5 mL) was then added and stirred overnight. The mixture was diluted in 0.5 M HCl/0.5 M NaCl (30 mL) and extracted with DCM (3 × 30 mL). The combined organic extracts were washed with the acid-brine solution (30 mL), dried ($MgSO_4$), filtered, concentrated under reduced pressure to a yellow-orange solution, and redissolved in methanol (15 mL). After sitting overnight at −20 °C, precipitated 3,5-dini-

Ser i+4: Ac-Ala-**Ser**-Ala-Ala-Ala-**Ser**-Glu-Ala-Ala-Ala-Lys-Glu-Ala-Ala-Ala-Lys-Ala-$CONH_2$

Dnb i+4: Ac-Ala-**Dnb**-Ala-Ala-Ala-**Dnb**-Glu-Ala-Ala-Ala-Lys-Glu-Ala-Ala-Ala-Lys-Ala-$CONH_2$

Dnb i+7: Ac-Ala-**Dnb**-Ala-Ala-Ala-Ala-Glu-Ala-**Dnb**-Ala-Lys-Glu-Ala-Ala-Ala-Lys-Ala-$CONH_2$

Dnb i+13: Ac-Ala-**Dnb**-Ala-Ala-Ala-Ala-Glu-Ala-Ala-Ala-Lys-Glu-Ala-Ala-**Dnb**-Lys-Ala-$CONH_2$

Aly i+4: Ac-Ala-**Aly**-Ala-Ala-Ala-**Aly**-Glu-Ala-Ala-Ala-Lys-Glu-Ala-Ala-Ala-Lys-Ala-$CONH_2$

FIGURE 1: Peptide sequences. Dnb = ϵ-(3,5-dinitrobenzoyl)Lys, Aly = ϵ-(acetyl)Lys.

trobenzoic acid was removed by filtration. The filtrate was concentrated under reduced pressure to ~4 mL, filtered again, and purified by reverse-phase HPLC in two injections using a LOBAR 310−25 mm LiChroprep RP 18 40−63 μm column (EM Industries) with a gradient of 50−90% methanol in water with 0.1% TFA at 10 mL/min over 30 min, with a 10 min wash of 90% methanol using UV detection at 305 nm. The broad peak eluting at 36.5 min was concentrated and lyophilized to afford the pure product as a light yellow precipitate (0.53 g, 0.94 mmol, 46%). Purity was confirmed by analytical reverse-phase HPLC using an isocratic mixture of 75% methanol in water containing 0.1% TFA at 1.0 mL/min, with UV detection at 305 nm. One peak, eluting at 8.8 min, was evident. mp 95−98 °C; ^{1}H NMR (300 MHz, DMSO-d_6) δ 9.05 (2H, s, 3,5-NBA), 8.89 (1H, s, 3,5-NBA), 8.19 (1H, s, NH), 7.85 (2H, d, 6.4 Hz, Fmoc arom), 7.67 (2H, d, 7.7 Hz, Fmoc arom), 7.35 (2H, m, Fmoc arom), 7.28 (2H, m, Fmoc arom), 4.36−4.14 (4H, m, Fmoc CH_2, Fmoc CH, αCH), 3.44 (2H, br s, ϵCH_2), 1.94 (1H, m, βCH_2), 1.78 (3H, m, βCH_2, δCH_2), 1.51 (2H, m, γCH_2); ^{13}C NMR (125 MHz, DMSO-d_6) δ 173.98, 161.85, 156.12, 148.06, 143.79, 143.67, 140.65, 136.99, 127.61, 127.36, 127.03, 125.29, 125.20, 120.64, 120.08, 65.58, 53.63, 46.62, 30.42, 28.31, 23.12; LRMS (FAB, MNBA/methanol) for $C_{28}H_{27}N_4O_9$ (M + H) 563. Anal. Calcd: C 59.78, H 4.66, N 9.96. Found: C 59.51, H 4.81, N 9.80.

Synthesis of ϵ-(Acetyl)-Fmoc-Lys (Fmoc-Aly). A solution of Fmoc-Lys (Bachem CA) (0.50 g, 1.35 mmol) in tetrahydrofuran (THF) (10 mL) was added to a solution of acetic anhydride (2.0 mL, 21.2 mmol) in 10% saturated sodium bicarbonate (40 mL) and THF (40 mL). After 15 min no free amines could be detected by ninhydrin. The reaction mixture was acidified by addition of concentrated HCl and concentrated under reduced pressure. The mixture was diluted with EtOAc (50 mL) and extracted with 0.1 M HCl (3 × 50 mL). The combined aqueous layers were washed with EtOAc (2 × 25 mL), and the combined organic layers were dried ($MgSO_4$), filtered, and concentrated under reduced pressure. The product was obtained as a white precipitate (0.41 g, 1.01 mmol, 74%) by crystallization from EtOAc/Et_2O. mp 142−144 °C; ^{1}H NMR (300 MHz, DMSO-d_6 and $CDCl_3$) δ 12.36 (1H, s, -COOH), 7.79 (2H, d, 6.4 Hz, Fmoc arom), 7.68 (2H, d, 6.5 Hz, Fmoc arom), 7.39 (2H, t, 6.9 Hz, Fmoc arom), 7.31 (2H, t, 7.2 Hz, Fmoc arom), 4.29 (2H, d, 6.7 Hz, Fmoc CH_2), 4.21 (1H, q, 7.23 Hz, Fmoc CH), 4.01 (1H, m, αCH), 3.07 (2H, br d, 5.67 Hz, ϵCH_2), 1.83 (3H, s, acetyl), 1.76−1.63 (2H, br m, βCH_2), 1.43 (4H, br m, δCH_2, γCH_2); ^{13}C NMR (125 MHz, DMSO-d_6 and $CDCl_3$) δ 172.26 (C), 167.34 (C), 154.40 (C), 142.04 (C), 138.97 (C), 125.77 (CH), 125.20 (CH), 123.46 (CH), 118.16 (CH), 63.89 (CH_2), 51.99 (CH), 44.97 (CH), 36.58 (CH_2), 28.78 (CH_2), 26.95 (CH_2), 21.32 (CH_2), 20.86 (CH_3); LRMS (FAB, MNBA/methanol) for $C_{23}H_{27}N_2O_5$ (M + H) 411.

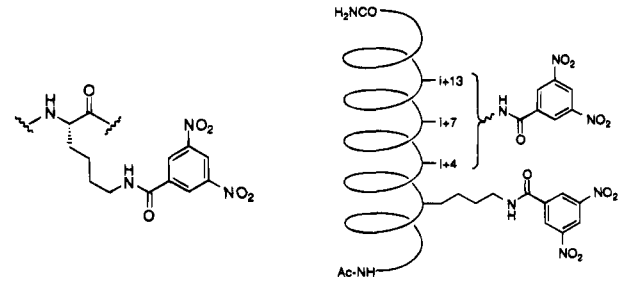

FIGURE 2: (Left) ϵ-(3,5-dinitrobenzoyl)Lys (Dnb). (Right) Schematic view of positioning of Dnb residues in helical peptides. In a perfectly helical conformation, the i+13 Dnb residue would be closer to the opposite side of the helix than indicated above.

RESULTS

Peptide Design. Peptides used in this study (Figure 1) are variants of a sequence originally designed by Baldwin (Marqusee & Baldwin, 1987) and employ several features commonly used to promote helix formation. Each peptide sequence has a high proportion of alanine and two stabilizing salt bridges, each between an i+4 Glu/Lys pair. The Glu and Lys residues are oriented to interact favorably with the helix macrodipole and minimize intermolecular aggregation. The helix termini are capped as their acetyl and amide derivatives. Similar peptides have shown that the amino terminus appears to be less frayed than the carboxyl terminus; therefore, the modified, hydrophobic amino acids are placed nearer the amino terminus to benefit from greater preorganization.

Hydrophobic Stabilization. To explore the role of hydrophobic interactions in structural stabilization, we employ a 3,5-dinitrobenzoyl derivative of lysine (Dnb) (Figure 2). The highly electron-deficient aromatic ring should promote stabilizing π/π interactions (Hunter & Sanders, 1990). To examine positional effects in these helical peptides, the Dnb i+4 and Dnb i+7 peptides have the aromatic residues on the same face of the helical peptide but are separated by a varying distance along the helix backbone (Figure 2). In the Dnb i+13 peptide, the aromatic residues are farthest apart and are on nearly opposite sides of the helix face. The Dnb i+4 peptide has the closest arrangement of hydrophobic groups and would be expected to benefit most from hydrophobic interactions. In the control peptide, Ser i+4, a hydrophilic, uncharged serine residue, is substituted for Dnb and therefore lacks any of the hydrophobic or π/π stacking interactions in the Dnb peptides. A second control peptide, Aly i+4, replaces the dinitrobenzoyl moiety with an acetamide from ϵ-acetyllysine (Aly). In this peptide, the potential for hydrophobic interactions between the lysine alkyl chains is maintained, but the aromatic interactions are absent.

All of these peptides have circular dichroism (CD) spectra with maxima at ~190 nm and minima at ~208 and ~222 nm (Figure 3), which are characteristic of α-helical conformations (Woody, 1985). These peptides are all highly

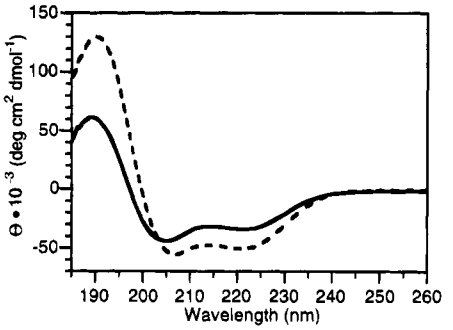

FIGURE 3: CD spectra of peptides Ser i+4 (solid line) and Dnb i+4 (dashed line) at 25 °C, in 90 mol % water (1.0 mM phosphate, 10 mM NaCl, pH 7.00) in TFE.

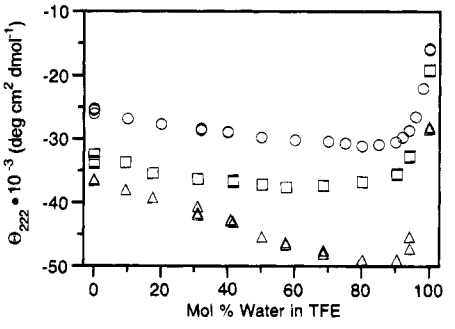

FIGURE 4: Solvent mixture titrations with water (1.0 mM phosphate, 10 mM NaCl, pH 7.00) in TFE at 25 °C, for peptides Aly i+4 (○), Ser i+4 (□), and Dnb i+4 (△).

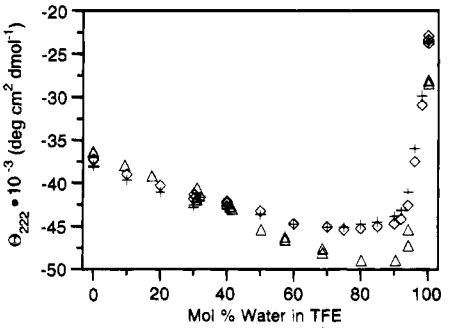

FIGURE 5: Solvent mixture titrations with water (1.0 mM phosphate, 10 mM NaCl, pH 7.00) and TFE at 25 °C, for peptides Dnb i+4 (△), Dnb i+7(◇), and Dnb i+13 (+).

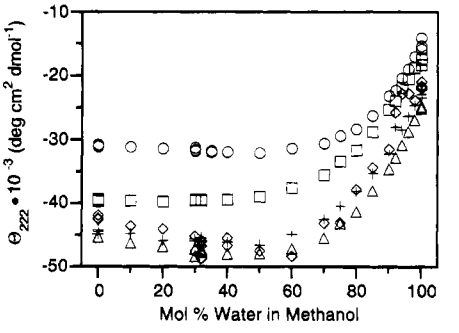

FIGURE 6: Solvent mixture titrations with water (1.0 mM phosphate, 10 mM NaCl, pH 7.00) and methanol at 25 °C, for peptides Ser i+4 (□), Aly i+4 (○), Dnb i+4 (△), Dnb i+7 (◇), and Dnb i+13 (+).

soluble in water, TFE, and methanol. There is no concentration-dependent change in the molar ellipticity, indicating that these peptides [like many related sequences (Marqusee & Baldwin, 1987)] remain monomeric over the concentration range 4-450 μM (data not shown).

To determine the dependence of helical stability as a function of solvent mixture, solvent composition titrations were carried out for each peptide over a range of 0−100 mol % water in TFE or methanol. Peptide Dnb i+4 shows considerable solvent-dependent changes in helical stability (Figure 4). Stability is favored more in neat TFE than in water but is highest in intermediate mixtures, with a maximum between 80 and 90 mol % water. The Ser i+4 and Aly i+4 peptides also have higher helical stability in pure TFE than in pure water; however, the structural stability is much less variant than the Dnb i+4 peptide between 0 and 80 mol % water.

If the high degree of helical stabilization in the Dnb i+4 peptide results from specific interactions between the hydrophobic groups, then the stability should be sensitive to the distance between them. Assuming a perfectly α-helical conformation, molecular modeling studies indicate that the distances between the laterally projecting Dnb side chains in each peptide are approximately the following: Dnb i+4, 6 Å; Dnb i+7, 11 Å; and Dnb i+13, >18 Å (Figure 2). Note, however, that the lysine alkyl chain allows considerable flexibility in the positioning of the aromatic residues. Consistent with the hypothesis of distance-dependent conformational stability, the Dnb i+7 and Dnb i+13 peptides show a much smaller increase in helical stability between 0 and 80 mol % water than the Dnb i+4 peptide (Figure 5).

Although the dinitrobenzoyl moiety has a strong UV absorption between 220 and 230 nm, the individual Dnb amino acid has a CD effect that is smaller than 2% (800 deg·cm^2·dmol^{-1}) of the maximum signal for the peptides. We cannot, however, discount the effect of an induced CD chromophoric change due to the dinitrobenzoyl group in these peptides. A similar (but small) effect has recently been observed by Baldwin due to the presence of a terminal tyrosine (Chakrabartty et al., 1993b). In our experiments, such an effect would result in a small displacement of the titration curve but would not dramatically alter its shape. Furthermore, the differences between the Dnb i+4 and Dnb i+7 peptides would not be expected, since each has the same number of modified residues and similar positioning. Our findings suggest that an induced chromophoric change due to the dinitrobenzoyl groups in the peptides cannot fully explain the solvent-induced stability effects described here.

We attempted to determine the maximum mean residue ellipticity for each of our peptides under optimal helix stabilizing conditions. At temperatures as low as −5 °C, we found the commonly observed effect of loss of melting cooperativity (resulting in a pseudolinear rather than sigmoidal CD melting transition) with no evidence of approaching convergence (data not shown). By interpolation, however, these results indicated a difference in thermal stability of greater than 40 °C between the Ser i+4 and Dnb i+4 peptides.

To further examine the role of TFE in helix stabilization, comparable solvent mixture titrations were carried out in methanol/water. Mixtures of methanol/water show significantly different helix-stabilizing properties than mixtures of TFE/water for these peptides. CD spectra indicate that each peptide is α-helical in these solvents (data not shown) and has nearly maximal helix content in neat methanol (Figure 6). For every peptide in this series, the degree of helix stability is relatively constant until ~60 mol % water is

reached; further addition of water results in helix unfolding. This is in contrast to TFE/water titrations that show significantly reduced helical stability in the neat alcohol, and maximum helix content at 80−90 mol % water.

The Ser i+4 peptide shows thermal denaturation behavior in the normal range expected for similar peptides. In such examples, it is generally observed that the maximum mean residue ellipticity at 222 nm (0−5 °C in water) is −30 000 to −40 000 deg·cm^2·dmol^{-1} and the high temperature minimum is between 0 and −6,000 deg·cm^2·dmol^{-1}. The ellipticity values for the Dnb peptides are significantly larger than those expected for peptides of similar length. In comparison to the Ser i+4 peptide, the Dnb and Aly peptides each have additional amide bonds that will contribute to the measured ellipticity. Simple normalization of these values to account for the additional amide bonds results in a 10% reduction of the observed ellipticity (the Ser i+4 peptide has 18 amide bonds while the other peptides have 20 amide bonds). This translates to a maximum observed mean residue ellipticity of −44 100, −40 860, and −40 590 deg·cm^2·dmol^{-1} for peptides Dnb i+4, Dnb i+7, and Dnb i+13, respectively, in mixtures of TFE/water at 25 °C. This estimation, however, is based on the assumption that the magnitude of the CD absorption from the side chain amides is the same as the backbone amides, which is unlikely to be exactly correct. For these reasons, it is difficult to precisely measure the helix content for the peptides.

DISCUSSION

The factors that stabilize helical conformations are important in the understanding of the mechanisms of protein folding. Numerous studies involving residue replacements (Chakrabartty & Baldwin, 1993; Chakrabartty et al., 1991; Gans et al., 1991; Lyu et al., 1990; Merutka et al., 1990; Merutka & Stellwagen, 1990; O'Neil & DeGrado, 1990; Padmanabhan et al., 1990) in related peptides have established scales of helical propensity for each amino acid. While these studies have been generally in close qualitative agreement and have provided valuable insight into the intrinsic helix-forming properties of the amino acids, they have not been much more useful in predicting protein helical structure than the Chou−Fasman statistical parameters (Chou & Fasman, 1974). A major difficulty in developing model peptides to study protein structure is the inclusion of the effects of longer range interactions (Shoemaker et al., 1985). In a study by Lehrman, it was attempted to correlate helical stability of peptide fragments of bovine growth hormone (in 90 mol % water with TFE) with the known crystal structure of the complete protein (Lehrman et al., 1990). Results indicated that most peptide segments which showed high helical stability in the mixed solvent correctly corresponded to helical domains in the protein. The degree of correspondence was similar to that predicted by the Chou−Fasman parameters. The most significant deviation, however, was for highly hydrophobic peptide sections that were significantly less helical than the corresponding domains in the protein crystal structure. This suggests that the overall contribution of factors that stabilize helical domains may be partially dependent upon the degree of favorable hydrophobic components.

TFE has frequently been used to stabilize helical domains in short peptides (Jasanoff & Fersht, 1994; Nelson & Kallenbach, 1986). Most examples show that helical stability increases with the addition of trifluoroethanol, having a plateau between 30 and 90 mol % water (Lehrman et al., 1990; Marqusee et al., 1989; Merutka & Stellwagen, 1989; Zhang et al., 1993). The reduced polarity of these solvent mixtures increases the stabilizing contribution of the hydrogen-bonding network but reduces the contribution of hydrophobic interactions. Recent theoretical results have offered some insight into the nature of helical stability which is promoted in aqueous−organic solvent mixtures (Brooks & Nilsson, 1993).

In a mixed solvent titration, the composition at maximum peptide stability must correspond to the optimal balance between electrostatic effects (favored in organic solvents) and hydrophobic effects (favored in water). In this study, the contribution of electrostatic stabilization should remain nearly constant among all peptides since they have identical lengths and dispositions of charged amino acids. Changes in peptide stability as a function of solvent must then be primarily due to the hydrophobic stabilization component.

The Ser i+4 peptide is typical in that helical structure is stabilized over a broad range of TFE concentrations. A small increase in helical stability is observed upon increasing the water concentration from 0 to about 60 mol %. This indicates a moderate hydrophobic contribution which is not surprising for a sequence that contains such a high proportion of alanine. Beyond 60 mol % water, the helicity rapidly decreases, consistent with a reduction in hydrogen bonding and salt bridge interactions. In contrast, the Dnb i+4 peptide shows a pronounced increase in helical stability up to about 80−90 mol % water. This effect indicates a larger stabilizing contribution due to hydrophobic interactions between the extended Dnb alkyl chains and π/π interactions of the aromatic substituents (Figure 7). The Dnb i+7 and Dnb i+13 peptides show a similar but smaller influence of hydrophobic stabilization, consistent with their less optimal arrangement of interacting side chains. Accordant with Dnb i+7 and Dnb i+13, the Aly i+4 peptide displays only a moderate increase in helical stability upon increasing the water concentration from 0 to 80% in TFE (Figures 4 and 5). This implies that the dominant factor in the hydrophobic stabilization component for the Dnb i+4 peptide originates from interactions between the dinitrobenzoyl groups, rather than between the lysine alkyl chains. Comparison of Dnb i+4 to the other Dnb peptides demonstrates the effect of position-dependent hydrophobic stabilization and precludes explanations simply due to induced chromophoric changes (Chakrabartty et al., 1993b) in the dinitrobenzoyl group.

Comparison of the TFE/water and methanol/water titrations suggests that the additional hydrophobic stabilization is significant only with TFE. Each of the peptides shows nearly maximal helical stability in neat methanol, with little change until the water concentration reaches ~60 mol %. In contrast, less helical stability is evident at high concentrations of TFE. The acidity of TFE [pK_a = 12.4 (Nelson & Kallenbach, 1986)] is considerably higher than that for methanol [pK_a = 15.5 (Perrin et al., 1981)]; therefore, TFE might be expected to destabilize the hydrogen bond network in the helix to a greater extent than methanol. Because methanol has nearly the same acidity as water [pK_a = 15.7 (Perrin et al., 1981)], the effective acidity of the peptide solutions remains approximately constant throughout the methanol/water titration. This is consistent with the broad

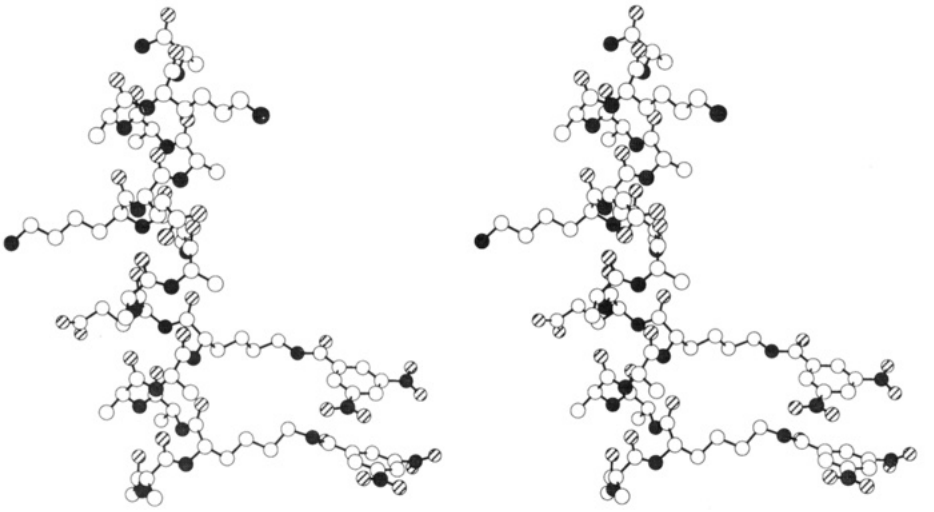

FIGURE 7: Stereoview of energy minimized model [Amber force field, Macromodel 3.5a (Mohamadi et al., 1990)] of peptide Dnb i+4. Hydrogens have been omitted for clarity.

range of helix stability observed in solutions of up to 60 mol % water in methanol. In contrast to the TFE/water titrations, there is no evidence for additional helix stabilization due to the hydrophobic modified amino acids. Helical stability in each of the Dnb peptides in methanol/water is approximately the same and shows no dependence upon the relative positioning of the two modified residues. This suggests that methanol is much more effective than TFE at solvating the hydrophobic components despite its slightly higher polarity [$\epsilon = 32.6$ for methanol (Gordon & Ford, 1972); $\epsilon = 26.67$ for TFE (Nelson & Kallenbach, 1986)]. Solvent mixtures of TFE/water therefore appear to be more effective in stabilizing hydrophobic interactions in these peptides.

Comparison of helical stability in each solvent mixture reveals some general trends. Maximum helicity is observed in intermediate mixtures of water in methanol, although helicity changes only slightly over the range of 0−60 mol % water (Figure 6). A similarly high degree of helical stability is observed in high percentage mixtures of water in TFE. In low-to-intermediate concentrations of water in TFE, helical stability increases significantly as water is added (Figures 4 and 5). This degree of variation is larger than that observed in methanol/water. The magnitude of the water-induced stability appears to correlate with the contribution of hydrophobic interactions in the folded peptide. This is evident for the Dnb i+4 peptide which shows the greatest increasing change in helical stability in comparison to its value in 100 mol % TFE. In all cases, helicity decreases dramatically as the concentration of water (in TFE or methanol) approaches 100 mol % and reaches its minimum value in neat water. The neat alcohols are more helix-stabilizing than neat water, with 100 mol % methanol being preferred over 100 mol % TFE.

Interestingly, our experimental results bear close relation to the theoretical results of Brooks and Nilsson (1993). They predicted the helix-promoting ability of various solvent mixtures in the following order: TFE/water ≈ methanol > methanol/water > water ≈ TFE. We find a qualitatively similar ordering of solvent mixtures with the most significant deviation being that helicity is found to be greater in 100 mol % TFE than in 100 mol % water. In the computational study, the anomalous behavior of TFE in TFE/water mixtures in promoting helix stability is attributed to ordering effects of the alcohol around the peptide in the presence of water. These effects would be expected to be magnified in our Dnb i+4 peptide, due to the highly hydrophobic interactions, thereby conferring the observed additional helical stability.

In summary, we have demonstrated the use of specific hydrophobic effects to stabilize helical domains in short peptides. Our results are consistent with the observed weakened effect of hydrophobic interactions in mixed solvents and suggest that full-range solvent mixture titrations in TFE/water will be useful in indicating the stabilizing effect of hydrophobic interactions. Studies are currently underway to examine more closely the contribution of hydrophobic stabilization using different aromatic components and variable length alkyl chains. Analysis of the thermodynamic parameters of denaturation of these and related peptides will be considered in a subsequent report.

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