Analysis of *i,i*+5 and *i,i*+8 Hydrophobic Interactions in a Helical Model Peptide Bearing the Hydrophobic Staple Motif

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ABSTRACT: In this work we have analyzed by far-UV circular dichroism the contribution to α-helix stability of pairwise hydrophobic interactions in the *hydrophobic staple* motif [Muñoz *et al.* (1995) *Nat. Struct. Biol. 2,* 380−385]. For this, we have used a new series of alanine-based model peptides having a *capping-box* motif (Ser-X-X-Glu) and no other charged residues to facilitate the determination of the interaction energies with a helix/coil transition algorithm. Our results show that the favorable *i,i*+5 interaction between a hydrophobic residue (Leu, Met, Ile, Val, Phe) at position N′ (before the N-cap) and a Leu at position N+4 (inside the helix) contributes up to −1.48 ± 0.18 kcal/mol to α-helix stability at 278 and pH 7. More interestingly, the same hydrophobic residues at position N′ interact favorably with an Ala at position N+4, although the interaction is weaker than that with Leu (up to −0.8 ± 0.14 kcal/mol at 278 K and pH 7). To our knowledge, this is the first example in which a strong pairwise interaction with Ala is described and suggests that Ala could be less neutral in terms of side chain−side chain interactions than normally assumed. We observe a strong stereospecificity for the position N′ which could be explained based on the extreme rigidity imposed by the formation in phase of the *hydrophobic staple* and *capping-box* motifs, as is seen in the protein structure database. We have also investigated the contribution to α-helix stability of a geometrically feasible *i,i*+8 hydrophobic interaction between residues N′ and N+7. In this regard, we find that most of the *i,i*+8 hydrophobic pairs have completely negligible interactions, while the Leu−Leu and perhaps the Ile−Leu pairs have a weak favorable *i,i*+8 interaction.

A complete understanding of helix formation and stability requires the quantification of all the possible interactions that could occur in this secondary structure element. Until very recently, mainly *i,i*+3 and *i,i*+4 side chain interactions, together with the intrinsic helical propensities of the various amino acids, were thought to play a role in helix stability [for a review, see Chakrabartty and Baldwin (1995)]. More recently, a preferential localization at the N- and/or C-termini of helices of specific groups of residues has been found, constituting local motifs (Dasgupta & Bell, 1993; Harper & Rose, 1993; Aurora *et al.*, 1994; Seale *et al.*, 1994; Muñoz *et al.*, 1995a). At the N-terminus, two local motifs have been described: the *capping-box*, which involves a reciprocal hydrogen bond between the main chain of the N-cap residue and the side chain of a Glu at position N3 [Harper & Rose, 1993; Dasgupta & Bell, 1993; see nomenclature of Richardson and Richardson (1988)], and the *hydrophobic staple*, which involves a hydrophobic interaction between the side chains of two residues located at positions N′ and N+4, plus a good N-capping residue (Ser, Thr, Asn, Asp) (Muñoz *et al.*, 1995a; Seale *et al.*, 1994). The *hydrophobic staple* motif is often (Seale *et al.*, 1994), but not necessarily, associated with the presence of a *capping-box* motif (Muñoz *et al.*, 1995a). At the C-terminus, the *Schellman* motif involves a residue with positive angles at position C′ and two main chain−main chain hydrogen bonds between residues C3 with C″ and C2 with C′ (Schellman, 1980). This motif is usually associated with the presence of a glycine residue at position *i*, a residue with a hydrophobic side chain at positions *i*−4 and *i*+1, and a polar or Ala residue at position *i*−2 (Aurora *et al.*, 1995; Viguera *et al.*, 1995).

Experimental analysis of designed peptides has shown that the *capping-box* (Lyu *et al.*, 1993) and *hydrophobic staple* (Jimenez *et al.*, 1994; Muñoz *et al.*, 1995a) motifs contribute significantly to α-helix stability, while the Schellman motif contributes significantly less (Viguera *et al.*, 1995). Moreover, the presence in phase of a *hydrophobic staple* and a *capping-box* motif seems to have a synergetic effect as observed in the statistical analysis of protein structure databases (Muñoz *et al.*, 1995a). Nuclear magnetic resonance (NMR)[1] analysis of a model peptide with both motifs in phase has shown that the hydrophobic side chain of the residue at position N′ is positioned to contact that of the residue at position N+4 (Muñoz *et al.*, 1995a). This interaction was found to contribute to α-helix stability when the residue at position N+4 is a Leu, although the quantification of the interaction energies was not carried out. The study also revealed a weak NOE between the side chain of a Phe at position N′ and the side chain of an Ala at position N+7. Observation of this NOE implies that the two side chains located at positions *i,i*+8 in the peptide sequence lie close in space and could have, as residues N′ and N+4, a pairwise interaction as a natural consequence of the formation of the *hydrophobic staple*. A similar interaction but between two oppositely charged residues was previously described, in the S-peptide of ribonuclease A, as being helix-stabilizing (Fairman *et al.*, 1990).

In this work, we have extended the experimental analysis of the hydrophobic interactions present in the *hydrophobic*

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[1] Abbreviations: CD, circular dichroism; NMR, nuclear magnetic resonance.

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staple motif (Muñoz *et al.*, 1995a). In particular, we have investigated the *i,i*+5 interaction between Leu and Ala at position N+4 and the different hydrophobic residues at N′, and the putative existence of an *i,i*+8 interaction between residues at positions N′ and N+7. Finally, the use of a different series of model peptides which do not include charged residues allowed the quantification of the interaction energies with a new version of the helix/coil transition algorithm AGADIR (Muñoz & Serrano, 1994, 1995a,b), which uses the multiple-sequence approximation and the same reference state for all residues (AGADIRms; Muñoz and Serrano, unpublished results).

EXPERIMENTAL PROCEDURES

Peptide Synthesis. All the peptides were synthesized with free ends, by the EMBL peptide service as previously described (Muñoz *et al.*, 1995a). The purity of the peptides was checked by HPLC chromatography and mass spectrometry.

Protein Database Analysis. The protein database used is implemented in the program WHATIF (Vriend, 1990) and has been published elsewhere (Muñoz & Serrano, 1994b). The conformational searches were carried out with the SCAN3D option of the same program using the Kabsch and Sander (1983) definition of secondary structure.

CD Analysis. The far-UV CD spectra were acquired in a JASCO-710 dichrograph, using the continuous mode with 1 nm bandwidth, 1 s response, and a scan speed of 50 nm/min, accumulating 30 scans. For each peptide, the experiment was repeated 3 times; the differences between them were within a ±3% margin, and their average spectrum was used for the analysis. The samples were prepared in 10 mM phosphate buffer, pH 7, and the experiments were carried out at constant temperature (278 K). The spectra used in the analysis were obtained at 25 μM peptide concentration in a 0.5 cm path length cuvette. All the peptides did not show changes in their spectral properties with peptide concentration in the range 5−500 μM. The peptide concentration was determined by the absorbance at 280 nm following the method of Gill and von Hippel (1989).

Calculation of the Helical Content. The helical content of the peptides was estimated by three different methods. The first one is based on the empirical equation developed by Chen *et al.* (1974):

$$\% \text{ helical content} = 100\{\theta_{obs}/[-39500(1 - 2.57/n)]\} \quad (1)$$

where n is the number of peptide bonds in the peptide and θ_{obs} is the experimentally observed ellipticity of the peptides at 222 nm. Since this method is extremely sensitive to the peptide concentration, we have also used the parameter $R1$ which is concentration independent (Bruch *et al.*, 1991). $R1$ is the ratio between the maximum ellipticity found within the range from 190 to 195 nm and the minimum ellipticity found within the range from 195 to 210 nm. This parameter changes as a function of the helix content and correlates well with the helix estimation from the ellipticity at 222 nm (Muñoz *et al.*, 1995b). In the third method, the helical content was estimated from the ellipticity at 193 nm using eq 2:

$$\% \text{ helix} = [(19.86 \pm 0.83) + \theta_{obs}(0.001 \pm 0.00005)] \quad (2)$$

where θ_{obs} is the mean residue ellipticity at 193 nm. This equation was obtained by correlating the ellipticity changes at 193 nm with the helical contents obtained from the method of Chen *et al.* (1974), for the peptides without Phe (Viguera & Serrano, 1995).

Calculation of the Energies of Interaction Using a Helix/Coil Transition Algorithm. Calculation of the energies of interaction between residues from changes in the helical content of short monomeric peptide requires a statistical mechanics approach (Chakrabartty & Baldwin, 1995). The reason is that a polyalanine-based peptide does not have a definite three-dimensional structure but an ensemble of many helical conformations. In this case, we have used the framework of the helix/coil transition model for heteropolypeptides termed AGADIR (Muñoz & Serrano, 1994b, 1995a,b), with a more precise and recent formulation that incorporates the multiple sequence approximation (AGADIRms; Muñoz and Serrano, unpublished results). In this new version, the molecular partition function also includes the molecular conformations with more than one nonoverlapping helical segment. The statistical weights of those conformations with more than one nonoverlapping helical segment are calculated as the product of the Boltzmann factors of the helical segments included on them. Helical populations of the residues in the molecule are finally normalized with the complete molecular partition function. This modification of the model does not affect the values for any of the energy contributions to α-helix stability previously reported (Muñoz & Serrano, 1995a), with the exception of the hydrogen bond/enthalpic contribution (from −0.775 to −0.794 kcal/mol). The incorporation in the model of the *i,i*+5 and *i,i*+8 interactions produced by the hydrophobic staple is straightforward. It is only necessary to include another two additive terms onto the analytic expression of the free energy of a helical segment previously developed (Muñoz & Serrano, 1995a):

$$\Delta G_{\text{hel segment}} = \Delta G_{int} + \Delta G_{Hbond} + \Delta G_{SD} + \Delta G_{nonH} + \Delta G_{dipole} + \Delta G_{i,i+5} + \Delta G_{i,i+8} \quad (3)$$

where, as previously described, ΔG_{int} represents the free energy required to fix a residue from a random coil conformation to a helical state, ΔG_{Hbond} is the energy contribution of main chain−main chain hydrogen bond formation, ΔG_{SD} represents the free energy contribution of *i,i*+3 and *i,i*+4 side chain−side chain interactions, and ΔG_{dipole} represents the interaction of charged groups with the helix macrodipole. $\Delta G_{i,i+5}$ accounts for the energy of interaction between residue N′ and residue N+4 and contributes to the stability of all the helical segments whose N-cap is the residue following residue N′, and $\Delta G_{i,i+8}$ accounts for the energy of interaction between residues N′ and N+7 and contributes to the stability of helical segments whose N-cap is the residue following residue N′ and which have a minimal length of nine residues (seven in helical angles, plus the N- and C-cap residues). In helical segments starting at the first position of the peptide, the two terms are set to 0 since residue N′ does not exist. This analytical expression is directly introduced in the statistical mechanics model with the multiple sequence approximation. The molecular partition function has only an extra modification to account for the fact that two helical segments (including caps) need at least one residue separating them to be

Table 1: Far-UV CD Analysis of the Different Peptides in Aqueous Solution

peptide[g]	ellipt(222 nm)[a]	min (nm)[b]	ellipt(193 nm)[c]	% helix(222 nm)[d]	% helix(193 nm)[e]	% helix(*R*1)[f]
AAA	−7551	204.4	4060	23.1	23.9	17.3
AAL	−7681	204.0	2819	23.5	22.7	17.1
VAA	−9456	205.1	8437	28.9	28.3	23.9
VAL	−9377	204.9	8629	28.6	28.5	24.3
VLA	−11638	205.8	16554	35.6	36.4	39.6
IAA	−10280	205.2	10231	31.4	30.1	27.0
IAL	−11518	205.6	12183	35.2	32.0	28.9
ILA	−12671	205.9	19718	38.7	39.6	44.8
LAA	−11383	205.6	16472	34.8	36.3	36.0
LAL	−13007	205.6	22410	39.7	42.3	47.4
LLA	−15577	205.9	26276	47.6	46.1	54.8
MAA	−13279	205.5	19899	40.6	39.8	45.4
MAL	−13586	205.7	19778	41.5	39.6	42.4
MLA	−16305	206.5	29277	49.8	49.1	60.3
FAA	−8362	205.1	12043	25.5	31.9	37.9
FAL	−7084	205.1	10533	21.6	30.4	31.6
FLA	−10542	206.0	24533	32.2	44.4	78.7

[a] Mean residue ellipticity in deg·cm^2·dmol^{-1} at 222 nm. [b] Position of the absolute minimum. [c] Mean residue ellipticity in deg·cm^2·dmol^{-1} at 193 nm. [d] Percent helical calculated from the ellipticity at 222 nm, using the method of Chen *et al.* (1974). [e] Percent helical content calculated from the ellipticity at 193 nm using eq 2 described under Experimental Procedures. [f] Percent helical content calculated from the *R*1 ratio (Bruch *et al.*, 1991; Muñoz *et al.*, 1995b). [g] The first letter corresponds to the residue at position X, the second to the residue at position Y, and the third to position Z of the template peptide shown under Results.

considered nonoverlapping (the N′ residue of the second helical segment), a requisite not necessary when the hydrophobic staple is neglected. Finally, the energy contribution for the interactions between different hydrophobic pairs is fitted to reproduce the average helical content experimentally observed by far-UV CD. The error margins on the determination of the interaction energies are obtained by fitting the model to reproduce the ±3% error found in the experimental determination of the peptide helical content.

RESULTS

Peptide Design. In this work, we have used a series of peptides, with the following template sequence:

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N" N' Ncap  N1  N2  N3 N4 N5 N6 N7 N8  N9.....
Gly-X--Ser--Ala-Ala-Glu-Y-Ala-Ala-Z-Gln-Ala-Ala-Ala-Gly-Tyr
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The nomenclature is that used by Richardson and Richardson (1988). The template peptide has been designed to have in phase the *capping-box* (Ser4 and Glu7) and the *hydrophobic staple* motifs, to increase the helical content due to their favorable synergetic activity (Muñoz *et al.*, 1995a). At the N-terminus, there is one Gly to prevent the helix-destabilizing effect of the N-terminal charged group (Muñoz & Serrano, 1994, 1995a,b). There is a Tyr residue at the C-terminus to determine peptide concentration, which is separated by one Gly residue from the rest of the peptide to minimize the contribution of the aromatic ring to the ellipticity at 222 nm (Chakrabartty *et al.*, 1993). This template sequence, different to the one previously reported (Muñoz *et al.*, 1995a), has the minimal number of charged residues in order to facilitate the quantification of the interaction energies (only the Glu necessary for the *capping-box* motif). At position X of the peptide (equivalent to N′), we have placed Gly, Ala, Val, Ile, Leu, Met, and Phe, and at positions Y (N+4) and Z (N+7), Ala or Leu.

Determination of the Average Helical Content from Far-UV CD. The average helical content of the peptides analyzed in this work has been determined by three methods (see Experimental Procedures). Helical contents calculated from the ellipticity at 222 nm and at 193 nm of peptides without Phe at position X are equal within ±1% (see Table 1). Those calculated with the *R*1 factor are also similar. The similarity is higher for peptides with helical contents between 30% and 40%, but this last method tends to underestimate peptides with contents lower than 30% and overestimate those with contents higher than 45% (Table 1). Such an effect is the result of the third-order polynomial dependence of the *R*1 parameter with the helical content (Muñoz *et al.*, 1995b). In the following discussion, since the helical content of the peptides studied varies from 20% to almost 50%, we use as experimental helical content the average between the values obtained at 222 nm and at 193 nm. For peptides with Phe at position X, the helical content calculated at 222 nm does not coincide with that calculated at 193 nm or with the *R*1 factor (Table 1). This effect arises from the conformational restriction of the Phe aromatic ring when the hydrophobic staple motif is formed (Muñoz *et al.*, 1995a), which results in a net contribution to the far-UV CD band (Woody, 1978; Chakrabarty *et al.*, 1993). However, the contribution is zero at 193 nm, and, therefore, the helical content of Phe-containing peptides can be determined with more precision from their ellipticity at this wavelength (Viguera *et al.*, 1995; see Experimental Procedure). Therefore, in the following discussion, the helical content for peptides with Phe refers uniquely to that calculated at 193 nm. In Figure 1, a correlation is shown between the values of the helical content calculated from the ellipticity at 193 and at 222 nm, for the whole set of peptides.

Analysis of the i,i+5 Interaction. Figure 2 shows the far-UV CD spectra of the peptides used to analyze the *i,i*+5 interaction. The peptide with three Ala at positions X,Y,Z (AAA) Figure 2A) presents a helical population of approximately 23% (Table 1). This peptide has been used as a negative control based on the assumption that the *i,i*+5 interaction between two Ala residues is negligible. The peptides with Leu at position Y (N+4) and the different hydrophobic residues at position X (N′) have a much larger helical content than the peptide AAA (Figure 2B−F; Table 1). A similar effect has been previously found in a different series of peptides and was assigned, on the basis of NMR

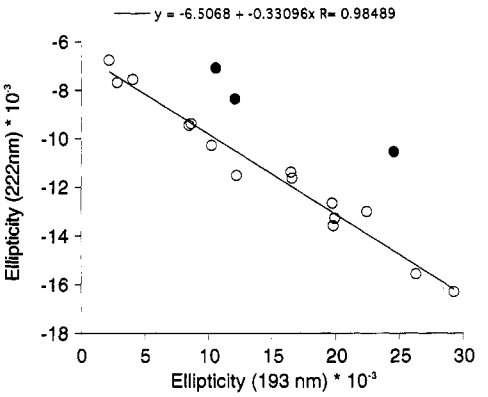

FIGURE 1: Comparison between the mean residue ellipticity at 193 and 222 nm. (Empty circles) Peptides without Phe. (Filled circles) Peptides with Phe.

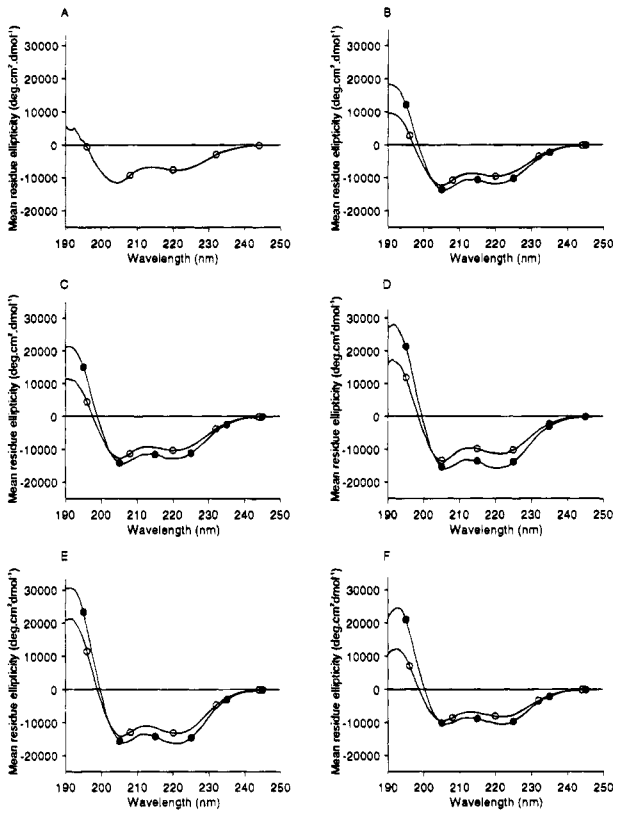

FIGURE 2: Far-UV CD spectra of the peptides used to analyze the *i,i*+5 interaction. (A) Peptide AAA. (B) Peptides VAA (empty circles) and VLA (filled circles). (C) Peptides IAA (empty circles) and ILA (filled circles). (D) Peptides LAA (empty circles) and LLA (filled circles). (E) Peptides MAA (empty circles) and MLA (filled circles). (F) Peptides FAA (empty circles) and FLA (filled circles).

analysis, to the presence of an *i,i*+5 pairwise interaction between the two hydrophobic residues (Muñoz *et al.*, 1995a). The interaction energy for the different *i,i*+5 hydrophobic pairs has been calculated with AGADIRms (see Experimental Procedures), assuming that the interaction of residue X (N′) with Ala at position Z (N+7) is negligible. The values calculated range between −0.45 ± 0.13 kcal/mol for the pair Val−Leu and −1.48 ± 0.18 kcal/mol for the Met−Leu pair (Table 2). This wide range reflects the stereospecificity of the *i,i*+5 hydrophobic interaction. More interestingly, we found that the presence of a hydrophobic residue at position X (N′) and an Ala at position Y (N+4) also renders a

Table 2: Free Energy Contribution to Helix Stability of the *i,i*+5 and *i,i*+8 Hydrophobic Interactions

peptide[a]	% helix$_{av}$[b]	ΔG (kcal·mol^{-1})[c]
VAA	28.6	−0.30 ± 0.14
VAL	28.5	0.0
IAA	31.0	−0.45 ± 0.13
IAL	33.9	−0.15 ± 0.12
LAA	35.6	−0.65 ± 0.15
LAL	41.0	−0.30 ± 0.14
MAA	41.0	−0.80 ± 0.14
MAL	40.6	0.0
FAA	32.4	−0.45 ± 0.14
FAL	30.7	0.0
VLA	36.0	−0.76 ± 0.12
ILA	40.0	−0.95 ± 0.13
LLA	45.0	−1.20 ± 0.15
MLA	51.4	−1.48 ± 0.18
FLA	46.7	−1.30 ± 0.15

[a] The first letter corresponds to the residue at position X, the second to the residue at position Y, and the third at position Z of the template peptide shown under Results. [b] Average helical content obtained from the values obtained using the ellipticity at 193 and 222 nm. [c] Free energy of interaction obtained by fitting the energy to reproduce the average helical content with AGADIRms (see Experimental Procedures). The error is obtained by calculating the free energy required to increase, or reduce, the average helical content in 3%. In those cases in which the difference between the peptides with Ala at position 10 and those with a Leu is less than 2%, we did not calculate the energies and assumed that they are null (−). To fit the data, we have considered that to fix an Ala in helical angles the energy cost is 0.64 kcal·mol^{-1}, for a Gly 2.02 kcal·mol^{-1}, and for a Gln 0.96 kcal·mol^{-1}. The energy contribution at the N-cap of a Gly is −0.5 kcal·mol^{-1} and at the C-cap −0.325 kcal·mol^{-1}. The hydrogen bond contribution is −0.792 kcal·mol^{-1}. The rest of the parameters are the same as the ones described in AGADIR (Muñoz *et al.*, 1995b).

significative increase of the helical content with respect to peptide AAA (Figure 2B−F and Table 1). Although the increase in helical content is smaller than for the peptides with Leu at position Y (N+4), it is still significant. Therefore, a hydrophobic residue before the Ncap (N′) also interacts favorably with residue N+4 when this is an Ala. We have calculated the interaction energies for the different pairs in the same way as above, obtaining values ranging from −0.3 to −0.8 kcal/mol (Table 2).

Analysis of the i,i+8 Interaction. A previous observation by NMR of a weak NOE between residues N′ and N+7 in a peptide with the *hydrophobic staple* and the *capping-box* motifs in register (Muñoz *et al.*, 1995a) suggests a possible *i,i*+8 pairwise interaction between them. To check whether two hydrophobic residues at positions N′ and N+7 have a helix-stabilizing interaction, we have also analyzed a series of peptides with the same template sequence, but in which the residue at position Z (equivalent to N+7) is a Leu and the different hydrophobic residues are placed at position X. The far-UV CD results show that the peptides with different hydrophobics at X (N′) and Ala at Z (N+7) (peptides XAA) have helical content equal, within experimental error, to the equivalent peptides of the series with Leu at position Z (peptides XAL) (Figure 3A−F and Table 1). The only peptides in which there seems to be a significant difference in the helical content are those having Ile (IAL)(Figure 3C) and Leu (LAL)(Figure 3D) at position X (N′). In both peptides, the helical content is slightly higher than in their respective controls (IAA and LAA). The energy of pairwise interactions for these two pairs has also been estimated in the same way as described above, obtaining values of −0.15

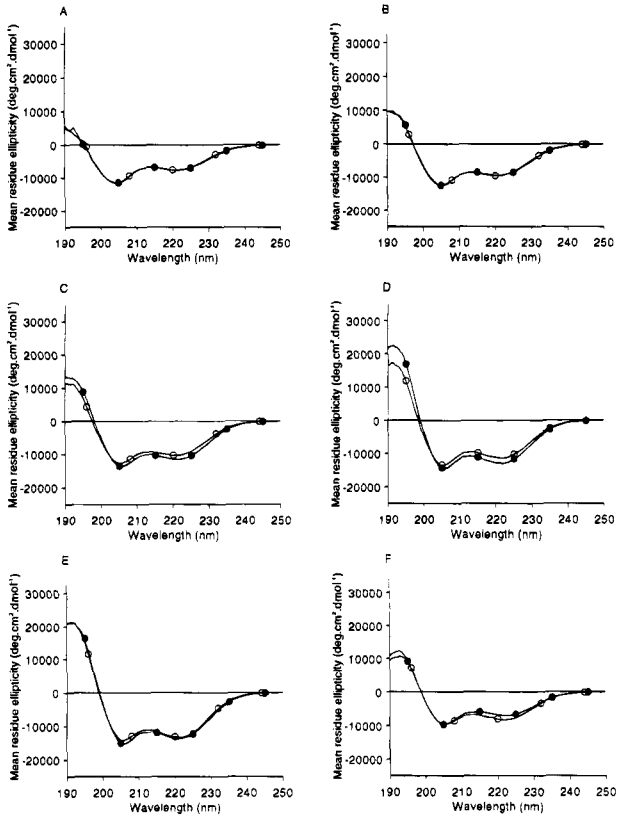

FIGURE 3: Far-UV CD spectra of the peptides used to analyze the *i,i*+8 interaction. (A) Peptides AAA (empty circles) and AAL (filled circles). (B) Peptides VAA (empty circles) and VAL (filled circles). (C) Peptides IAA (empty circles) and IAL (filled circles). (D) Peptides LAA (empty circles) and LAL (filled circles). (E) Peptides MAA (empty circles) and MAL (filled circles). (F) Peptides FAA (empty circles) and FAL (filled circles).

± 0.10 for the pair Ile−Leu (almost negligible) and −0.3 ± 0.12 for the Leu−Leu pair.

DISCUSSION

The hydrophobic staple motif consists of an *i,i*+5 interaction between two bulky hydrophobic residues placed at the position preceding the Ncap and at position N+4 inside the α-helix.

In this work, we present the quantification of the free energy of interaction for all the pairs with Val, Ile, Leu, Met, or Phe at position N′ and a Leu at position N+4. A precise quantification has been possible after the utilization of a series of peptides with a new template sequence that does not include bulky charged residues (Arg and Lys), which complicate the analysis. These energies tend to be very large in all cases, especially for the Leu−Leu and Met−Leu pairs, if we compare them with the energies that seem to be involved in *i,i*+3 and *i,i*+4 interactions within the α-helix (Muñoz and Serrano, 1994b, 1995a; Chakrabartty & Baldwin, 1995). Even more remarkably, we find that *i,i*+5 interactions between a bulky hydrophobic residue with an Ala at position N+4 are also helix-stabilizing and have important interaction energies (up to −0.8 kcal/mol), although less than with Leu. This interaction is geometrically feasible, and importantly it suggests that Ala could be less neutral in terms of side chain−side chain interactions than normally assumed.

Another interesting observation that arises from this work is the strong stereospecificity found for the *i,i*+5 interaction

Table 3: Analysis of the Dihedral Angles and Side Chain Rotamers in Protein Capping-Boxes[a]

protein	finger-print	N′			N-cap			N+1		N+2		N+3			N+4	
		ϕ	ψ	χ^1	ϕ	ψ	χ^1	ϕ	ψ	ϕ	ψ	ϕ	ψ	χ^1	χ^2	χ^3	ϕ	ψ
1btl (92−97)	MTXXEL	−142	153	−67	−84	165	58	−56	−42	−58	−46	−67	−34	−70	158	10	−73	−39
1rec (104−109)	ISXXEV	−101	132	−61	−103	167	70	−60	−40	−65	−32	−68	−43	−65	177	−45	−61	−43
4icb (24−29)	LSXXEL	−96	131	−62	−82	171	62	−61	−42	−63	−40	−64	−38	−71	161	−18	−65	−43
2dkb (411−416)	VSXXEI	−57	139	69	−90	164	84	−57	−44	−62	−45	−61	−40	−65	162	−9	−63	−43
1rec (15−20)	FTXXEL	−138	155	−66	−98	168	63	−60	−35	−68	−42	−69	−38	−65	173	−21	−65	−38
2cmd (194−199)	FTXXEV	−129	142	−56	−71	164	70	−67	−28	−71	−42	−62	−43	−76	165	−7	−58	−45
1gdl (251−256)	VTXXEV	−130	170	−69	−114	164	69	−62	−37	−70	−35	−72	−42	−42	164	26	−59	−52
1osa (116−121)	LTXXEV	−104	142	−61	−71	168	66	−65	−30	−67	−40	−62	−48	−70	177	−43	−66	−36
1rec (154−159)	FTXXEV	−114	125	−172	−91	166	69	−62	−37	−58	−53	−65	−40	−59	178	79	−58	−48
3grs (365−370)	LTXXEA	−82	145	−55	−78	169	65	−58	−41	−65	−42	−63	−37	−71	165	−22	−71	−39
1mba (2−7)	LSXXEA	−101	163	−87	−81	168	67	−60	−43	−61	−45	−66	−41	−69	166	−27	−61	−43
1phb (224−229)	ITXXEA	−61	151	−159	−83	171	72	−70	−38	−66	−43	−66	−39	−60	165	−18	−70	−38
451c (66−71)	VSXXEA	−124	154	−73	−88	170	71	−58	−43	−69	−30	−71	−39	−71	170	−23	−66	−38
31zm (58−63)	ITXXEA	−128	171	63	−97	168	68	−58	−46	−55	−46	−66	−39	−77	171	−13	−62	−44
1brn (23−28)	ITXXEA	−134	148	56	−73	170	67	−61	−44	−60	−41	−67	−45	−69	170	−25	−61	−43
1ede (198−203)	LTXXEA	−67	145	−66	−86	172	69	−60	−40	−61	−46	−61	−46	−69	170	−22	−60	−41
3tgl (5−10)	ATXXEI	−79	148		−83	164	73	−59	−48	−61	−46	−64	−40	−83	171	−40	−64	−38
average[b]		−106 ± 26	149 ± 12		−87 ± 13	168 ± 3	68 ± 5	−62 ± 4	−39 ± 5	−64 ± 5	−42 ± 5	−66 ± 3	−41 ± 4	−68 ± 9	169 ± 6		−64 ± 4	−42 ± 4

[a] The protein database was searched for the following fingerprint sequence: H-S/T-X-X-E-H (where H is Ala, Val, Ile, Met, Leu, or Phe), in which the two three amino acids are in nonhelical conformation, while the remaining four are helical, the side chain of the Glu at position N+4 is hydrogen bonded to the main chain amide of the Ser, and the hydrophobic residues are in van der Waals contact. [b] Average values obtained from the corresponding columns. The errors correspond to the standard deviation, and when it is higher than the average value, we do not show the data.

in the different pairs of hydrophobic residues. Particularly, the pair Val−Leu interacts with half the intensity of a Met−Leu pair (see Table 2). This is really interesting because hydrophobic interactions are assumed to be not very specific in peptides, in which there is no tight packing of side chains as occurs in proteins (Muñoz *et al.*, 1995b). A possible explanation for these observations is that the combination of the *hydrophobic staple* and the *capping-box* reduces the conformational flexibility in the region inasmuch as it constrains the geometry of the interaction. In fact, a protein structure database search for helices with a *capping-box* and *hydrophobic staple* motifs in phase (fingerprint sequence H-S/T-X-X-E-H, where H is Ala, Val, Ile, Met, Leu, and Phe) shows that the dihedral angles for the six residues as well as the rotamers of the N-cap (Ser or Thr) and Glu side chains are very restricted (Table 3).

The last important point of this work refers to the putative $i,i+8$ pairwise interaction between two bulky hydrophobic residues at positions N′ and N+7 of the α-helix. A similar $i,i+8$ interaction, between two hydrophobic residues in a helical peptide, was originally found by Merutka *et al.* (1993). The authors interpreted it as proof of the existence of conformations alternative to α-helices. More recently, it has been shown that the $i,i+8$ interaction could be geometrically compatible with an α-helix, when the capping-box and the hydrophobic staple motifs are present (Muñoz *et al.*, 1995a). In this study, we have analyzed the contribution of this putative interaction to α-helix stability, and we have found that it does not contribute significantly for most of the residue pairs. Only the pair Leu−Leu, and perhaps the pair Ile−Leu, produces an observable increase in the helical content as would be expected from a favorable interaction. The interaction energy for the Leu−Leu pair has been estimated at -0.3 ± 0.12 kcal/mol, which means a weak interaction, although similar to those found in some side chain−side chain $i,i+3$ and $i,i+4$ interactions (Muñoz *et al.*, 1995b). It is possible that the $i,i+8$ interaction observed in these two pairs is a side effect of the geometrical rigidity produced by the combination of the *hydrophobic staple* and *capping-box* motifs. On the other hand, in a survey of protein structure databases, we have found several examples of α-helices without the capping-box motif but with two hydrophobics at N′ and N+7 positions with van der Waals contacts (data not shown), indicating that the interaction is feasible even without the *capping-box* motif.

CONCLUSIONS

In this work, we have determined the interaction energies of several hydrophobic pairs in the *hydrophobic staple* motif combined with the *capping-box* motif. The interactions are strong, even for alanine, and rather stereospecific, contrary to what would be expected from hydrophobic interactions in peptides. These results highlight the interest of studying in the future the same hydrophobic interactions in the absence of the capping-box motif, to determine the extent of their synergetic effect.

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