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Homochiral Stereochemistry: The Missing Link of Structure to Energetics in Protein Folding

Anil Kumar,[†] Vibin Ramakrishnan,[‡,§] Ranjit Ranbhor,[‡,||] Kirti Patel,[†] and Susheel Durani*[,†]

Department of Chemistry and School of Biosciences and Bioengineering, Indian Institute of Technology Bombay, Mumbai 400076, India

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The notion is tested that homochiral stereochemistry being ubiquitous to protein structure could be critical to protein folding as well, causing it to become frustrated energetically providing the basis for its solvent- and sequence-mediated control. The proof in support of the notion is found in a consensus of experiment and computation according to which suitable oligopeptides are in their folding−unfolding equilibria, at both macrostate and microstate levels, susceptible to dielectric because of the conflict of peptide-chain electrostatics with interpeptide hydrogen bonds when the structure is poly-L but not when it is alternating-L,D. The argument is thus made that homochiral stereochemistry may in protein folding provide the unifying basis for its solvent- and sequence-mediated control based on screening of peptide-chain electrostatics under conflict with folding of the chain due to homochiral stereochemistry. Dielectric is brought into spotlight as the effect comparatively obscure but presumably critical to the folding in protein structure for its control.

Introduction

The folding of proteins in two-state equilibrium implies that their chain segments are so conflicted in the interactions among themselves and with solvent that the maximally solvated and desolvated states of the chain are the only participants in the equilibrium. Furthermore, the folding being under sequence control[1] implies that critical to arbitration in the conflicted interactions of the chain is the role of side chains. The conflict and its arbitration are unclear in their basis as part of the long-standing puzzle called the protein-folding problem. An important interaction of chain segments in folded protein is the hydrogen bonds defining secondary structure.[2,3] Despite earlier views to the contrary,[4] the hydrogen bonds contribute favorable enthalpy in chain folding.[5–8] Sequence defines the folds of secondary structure, but no hypothesis for their side-chain-mediated control has met sustained consideration. Crucial to protein folding is the solvophobic aggregation of nonpolar side chains.[9,10] Thus, while polarity in side chains will contribute to folding, it does not explain secondary-structure propensities being unrelated to side-chain polarity.[11] If hydrogen bonds of main chain and solvophobic interactions of side chains fold proteins, then a countervailing effect comparable in magnitude must unfold them in order for the equilibrium to be two-state. The strength of solvation of peptide chain may not characterize the effect unfolding proteins according to a recently reported computational study.[12] The polymer entropy of the unfolded chain, presumed to be a random coil,[13] has been considered as the dominant effect unfolding proteins. However, on much recent evidence, proteins are found to unfold largely to the PPII subzone of the β basin in the Ramachandran ϕ,ψ map[12,14,15] rather than to the entirety of its allowed zones as required for a random coil. Accordingly, it is unclear if the gain in polymer entropy accompanying protein unfolding will adequately compensate the loss of hydrogen bonds of main chain and solvophobic aggregates of side chains explaining two-state equilibrium. Clearly, protein folding remains in the dark about the energetics of its solvent- and sequence-mediated control.

Here, we test stereochemistry if it may provide the energetic link between structure and conformation in protein folding. Proteins come to be defined as much with the chemical alphabet in side chains as with attachment stereochemistry of the alphabet with main chain. The attachment is biased biologically to poly-L, viz., homochiral structure, which has firm acceptance as being the consideration defining both the conformational space[16,17] and the secondary structure[2,3] in protein folding. The question begs an answer: does it also define the protein-folding physics? As proteins fold by minimizing free energy,[1] the physics is in the microscopic detail of folding−unfolding equilibrium. The equilibrium may be modeled with statistical mechanics, but modeling explicitly the involvement of solvent makes the computation difficult. A way out may involve the simulation of folding with possible minimal protein models. Oligoalanines are arguably the smallest protein models emulating "folded"[18] and "unfolded" proteins[14,19] depending on the chain length. Seeking to model "unfolded" protein using a shorter alanine peptide, we adopted Ac-Ala$_8$-NHMe, expecting it, given the reports of Kallenbach and co-workers for their similar peptide XAO of seven alanines end-capped with pairs of cationic residues,[14,19] to be an ensemble of polyproline-II (PPII) helices. Contrarily, our "unfolded" protein model turned out to be "folded" in water as an ensemble of hairpins[20] and "unfolded" in methanol as an ensemble of PPII helices.[21,22] Other reports appeared in the meanwhile showing that XAO, while PPII helical in its macrostate, was an ensemble of not only "extended" but also "folded" microstates[23–25] and thus in a protein-like equilibrium, "folding" and "unfolding" dynamically.

Testing if in this mimicry of protein folding Ac-Ala$_8$-NHMe may implicate a role for stereochemistry, we found the peptide

* Corresponding author. E-mail: sdurani@iitb.ac.in. Phone: +91-22-25767164. Fax: +91-22-25767152.

[†] Department of Chemistry.

[‡] School of Biosciences and Bioengineering.

[§] Present address: Institute of Bioinformatics and Applied Biotechnology, ITPB, Bangalore 560066, India.

[||] Present address: Computation & Informatics, Sphaera Pharma Pvt Ltd, Manesar, Haryana 122051 India.

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losing its ability to fold and unfold when mutated from poly-L to alternating-L,D structure.[21,22] Extending the observation, we now show that even Ac-Lys-Ala_3-Lys-Ala_3-Lys-NH_2, the shorter length oligoalanine solubilized by Lys residues, clearly is a two-state folder, and specifically because of the homochiral structure in the interactions among its dipoles, since with the dipoles interacting in heterochiral structure the alternating-L,D peptide becomes impervious to solvent. The conclusion we draw is that proteins may in their two-state equilibrium manifest the role of homochiral stereochemistry in promoting a conflicted electrostatics, providing for folding the control principle based on screening of electrostatics being frustrated due to stereochemical reasons. The two-state equilibrium may thus involve as its antagonistic forces not the solvent entropy of hydrophobic effect against the polymer entropy of random coil but the mutual hydrogen bonds of peptide dipoles against their mutual electrostatics, providing the rationale for solvent- and sequence-mediated control of folding as well. There have been numerous studies of the folding of peptide-chain structure in the gas phase[26–30] with relevance for important experimental manifestations[31–36] such as amyloidogeneis.[31] In parallel, there have been numerous theoretical studies of solvation of the poly-L peptide chain structure[12,37–43] implicating for it a critical role in the observations, like secondary-structure propensities of side chains,[38,40,42] side-chain effects on NMR J values,[37] and α-proton chemical shifts,[41] with bearing on the sequence-mediated control of conformation. Summarizing the reports, we argue in the light of the present results that homochiral stereochemistry could in protein folding provide the unifying basis for its solvent- and sequence-mediated control, involving the electrostatics of homochiral-chain structure and its screening as the basis for the control. The proposed model brings dielectric into the spotlight as the effect comparatively obscure but presumably critical to the folding in protein structure for its control.

Results

Experimental Studies of Folding−Unfolding Equilibria with a Nona-Peptide Model. Aiming to combine experiment with theory in enquiry of principles, Ac-Lys-Ala_3-Lys-Ala_3-Lys-NH_2 was targeted as the protein model with protonable lysines capable of solublizing it. The diastereomers with all residues L or four of alanines D were made to poly-L and alternating-L,D structures. The syntheses are described under Materials and Methods, while the validations of purity and identity of the peptides are described under the Supporting Information. The peptide conformational equilibria were evaluated macroscopically with NMR, CD, and microcalorimetry. $^3J_{NH-C\alpha H}$ coupling constants will report on ensemble-averaged ϕ values,[44] amide-NH temperature coefficients on average solvent access in amide protons, CD on possible specific folds of polypeptide structure,[45] and microcalorimetry on possible thermal induced cooperative transitions. There are small variations in $^3J_{NH-C\alpha H}$ and amide-NH temperature coefficients in our peptides, as noted in Table 1 and Figures S3−S5 of the Supporting Information. No specific NH resonance could be assigned due to overlapped C_α−H resonances of the peptides. Hence, should any position-specific folds exist, they cannot be characterized on the basis of NMR. The somewhat differentiated J values of the peptides imply that if poly-L isomer is PPII in its average conformation (requiring ϕ to be −75° and correspondingly $^3J_{NH-C\alpha H}$ to be 6.1 Hz according to the Karplus relation; the observed range is 5.6−6.4 Hz) similar to XAO,[14,19] alternating-L,D peptide has average ϕ's of > −75° given the wider spread in J's (5.6−8.0 Hz). All that can be inferred broadly is that, if averaging along the chain, the poly-L isomer would be a PPII helix, much as reported for XAO. In contradiction of the interpretation, the CD results show that the poly-L peptide is in two-state equilibrium as implied in the appearance of an isodichroic point in temperature and solvent variation experiments, more clearly in the former, as noted in Figure 2. The peptide could be "disordering" or "unfolding" in a two-state transition with increase in temperature and similarly on increasing the proportion of methanol in water. Stereochemistry may have a role, as the alternating-L,D peptide apparently is inert to solvent according to CD. However, being antipodal in its alternating residues and thus compromised in molecular chirality, the alternating-L,D peptide may be CD opaque for this reason. We test the isomeric peptides with differential scanning calorimetry (DSC) as an opportunity to observe thermally induced folding−unfolding equilibria directly. According to the results in Figure 3, there is a feeble but definitive transition observable with the poly-L peptide, it is reversible, as could be expected for a two-state system, and it has a midpoint (~61 °C) surprisingly high for a system so small. The alternating-L,D peptide, while displaying thermal transitions in a comparable temperature zone, is rather complex and irreversible in the pattern, as can be noted in Figure 3. The alternating-L,D peptides are known to self-associate as parallel and antiparallel dimers in β-sheet-like intermolecular hydrogen bonds and intertwine as double β-helices observable with NMR.[46] Presumably, alternating-L,D peptides will self-associate as a rule unless specific measures are taken to prevent it. We interspersed our

TABLE 1: $^3J_{NH-C\alpha H}$ Coupling Constants and Amide-NH Temperature Coefficients of Poly-L and Alternating-L,D Isomers of Nona-Peptide (Ac-Lys-Ala_3-Lys-Ala_3-Lys-NH_2)

increasing order of chemical shift	$^3J_{NH-C\alpha H}$		amide-NH temperature coefficients (ppb/K)	
	poly-L	Alt-L,D	poly-L	Alt-L,D
1	5.6	6.4	7.2	5.6
2	6.4	8.0	7.4	6.1
3	5.6	6.4	7.3	5.9
4	5.6	7.2	7.4	7.4
5	5.6	6.4	7.1	7.4
6	*a*	5.6	7.5	6.5
7	*a*	6.4	7.2	7.3
8	*a*	6.4	*a*	6.8
9	*a*	5.6	*a*	8.1

[a] Not observed due to resonance overlap.

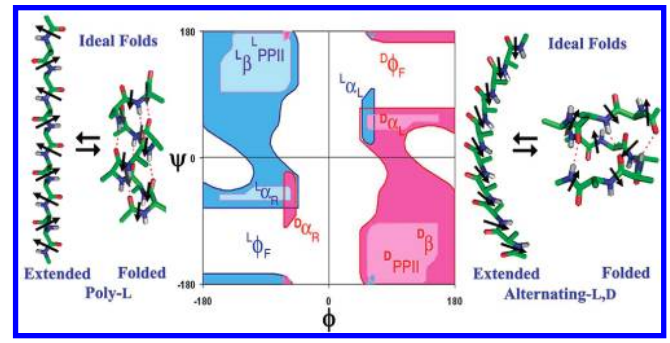

Figure 1. Constrained to only the blue (poly-L) or red (poly-D) zones of ϕ and ψ, the folding of homochiral polypeptide structure to the $^L\alpha$ or $^D\alpha$ basins is driven by the hydrogen bonds of the peptide dipoles, while its unfolding to $^L\beta + {}^L$PPII or $^D\beta + {}^D$PPII basins is driven by the electrostatics of the peptide dipoles mutually antiparallel. The energetic frustration is stereochemical in its basis, as the folding of the alternating-L,D peptide structure to the alternating $^L\beta$, $^D\beta$ basins, i.e., the blue and red zones of ϕ and ψ, is driven by both hydrogen bonds and electrostatics of the peptide dipoles.

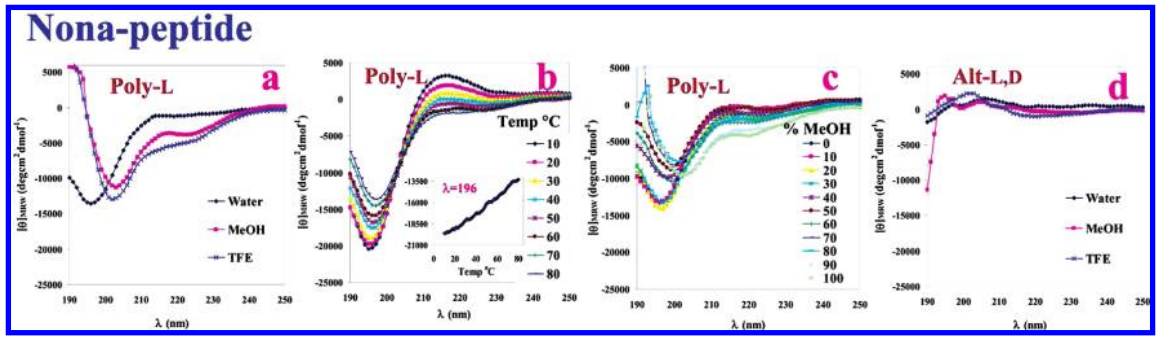

Figure 2. CD spectra of Ac-Lys-Ala$_3$-Lys-Ala$_3$-Lys-NH$_2$ showing the solvent (panels a and c) and temperature dependence of the folding−unfolding equilibria (panel b) of the poly-L peptide, against the apparent solvent insensitivity of the alternating-L,D isomer (panel d).

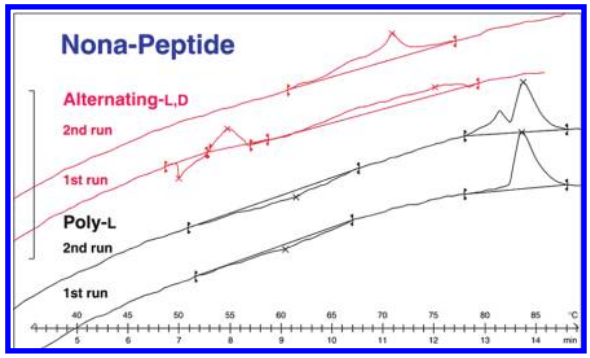

Figure 3. DSC traces of nona-peptide (Ac-Lys-Ala$_3$-Lys-Ala$_3$-Lys-NH$_2$) showing the contrasted melts of the poly-L and the alternating-L,D isomers.

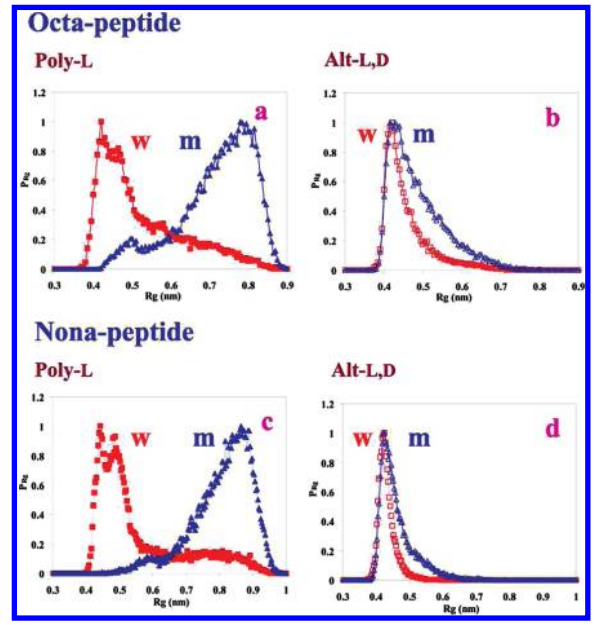

Figure 4. Macrostates of Ac-Ala$_8$-NHMe (octa-peptide) and Ac-Lys-Ala$_3$-Lys-Ala$_3$-Lys-NH$_2$ (nona-peptide), prepared with molecular dynamics, have the poly-L isomers apparently folded or unfolded based on solvent [water (w) or methanol (m)] (panels a and c) and the alternating-L,D isomers apparently folded in indifference of solvent (panels b and d), as judged with the radius-of-gyration (R_g) distribution over all of the peptide conformers populating the macrostates.

nona-peptide with cationic Lys residues specifically to prevent the possibility for self-association. The peptide was monomeric, as could be judged from concentration invariance of its chemical shifts and line widths in NMR (data not shown). However, the possibility remains that the associative propensity of the alternating-L,D structure manifests as the peptide unfolds, providing a plausible explanation for its complex and irreversible transitions observed with DSC.

Statistical−Mechanical Modeling of Folding−Unfolding Equilibria in Octa- and Nona-Peptides. We generated canonical ensembles in nonameric Lys peptide and octameric Ala peptide (Ac-Ala$_8$-NHMe), using water and methanol as solvents, knowing them to promote in our homochiral octa-peptide the "folded" and "unfolded" states. The ensembles were prepared with molecular dynamics as described under Materials and Methods and over the duration to attainment of equilibrium (∼250 ns) as could be judged from the ensembles becoming asymptotic in the growth of microstates, being the peptide structures clustered to 0.15 nm rmsd over main chain (results in the Supporting Information, Figure S7). The equilibria were analyzed on the basis of the radius of gyration over peptide structure for the state of "folding" or "unfolding". We find our poly-L isomers apparently "folded" in water and "unfolded" in methanol (Figure 4a,c) in conformity with the consensus of experiment with our Lys peptide. Stereochemistry is critical, as alternating-L,D isomers are apparently "folded" irrespective of the solvent (Figure 4b,d). Being independent of side chains, the selectivity of solvent role is an effect of the main-chain structure in both of the peptides.

Resolving the canonical ensembles to the microstates we analyzed the effects of stereochemistry and solvent. Based on the results (Figure 5a,c; Table S3 and Figures S8 and S9 of the Supporting Information) we found the microstates of poly-L structure "folded" in water, mostly as hairpins apparently maximized in intrachain interaction, and "unfolded" in methanol, mostly as PPII helices apparently maximized in chain-solvent interaction. Stereochemistry quite clearly is the reason as the microstates of alternating-L,D structure are "folded" to apparent maximization of intrachain interaction irrespective of the solvent (Figure 5b,d). The contrasted solvent effect, being independent of side chains, is an attribute of the main-chain structure in the isomers. Counterintuitively, the poly-L peptides "fold" to an increased density of microstates (Table 2); viz., they become disordered on the adoption of folded conformation. Stereochemistry quite clearly is the reason, as alternating-L,D peptides are in the density of microstates practically impervious to solvent (Table 2). Thus, it is noteworthy that the "two-state" equilibrium in our "protein" model is not an "order-to-disorder" transition, the hallmark of protein folding, but a "folded-to-unfolded" transition; the "states" under equilibrium are contrasted in not the degree of "order" but the degree of "solvation".

The poly-L microstates, "unfolded" predominantly in β+PPII ϕ,ψs, "fold" with dispersal to α-helix-and-the-turn-and-loop-specific ϕ,ψs in α_R (ϕ_F) and α_L (ϕ_U) basins of the ϕ coordinate, comprising the sterically favored (ϕ_F) and unfavored (ϕ_U) halves of the coordinate (see Figure 1, Table 2, and Figure S8 of the Supporting Information). The dispersals to the ϕ,ψs disfavored

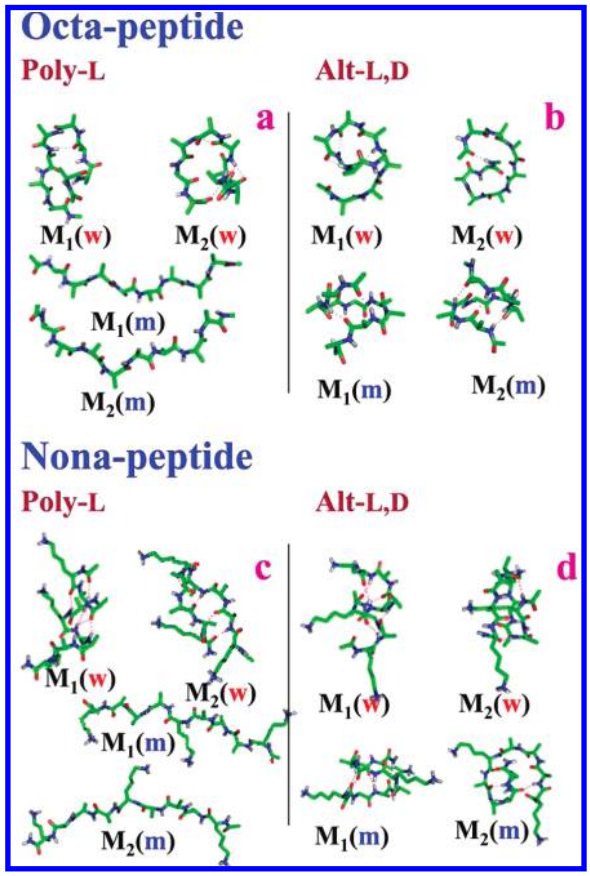

Figure 5. The two most populous microstates (M_1 and M_2) of octa-peptide (Ac-Ala_8-NHMe) and nona-peptide (Ac-Lys-Ala_3-Lys-Ala_3-Lys-NH_2) are folded or unfolded in the poly-L isomer depending on solvent [water (w) or methanol (m)] (panels a and c) but folded in the alternating-L,D isomers irrespective of the solvent (panels b and d).

electrostatically (in α_R and α_L basins)[47] and sterically in (α_L basin)[16,17,48] could cause the folding to become frustrated, and quite clearly for stereochemical reasons, as the alternating-L,D peptides will fold without being dispersed out of the β+PPII basin of the preferred halves in ϕ, i.e., ϕ_F (Table 2 and Figure S8 of the Supporting Information). We need to consider if stereochemistry may define the protein-folding physics in the energetic frustration of the polypeptide structure homochiral in stereochemistry.

Computing the Energetics in Folding−Unfolding Equilibria. We analyze the energetics of folding−unfolding transitions for the dependence on stereochemistry. As the effects belong to the peptide main chain, we perform this analysis with only Ala peptides (Table S1-S2 of Supporting Information). Focusing on the top microstates (M_1) in water (w) and methanol (m) and the "ideal-folds folded" (IF_F), we compute the folding energies from the "ideal-folds extended" (IF_E). Our "ideal folds" modeled in Figure 1 are noted to be "folded" or "extended" and with peptide-dipole vectors parallel or antiparallel depending on stereochemistry, which in residue-level structure defines its symmetry in ϕ,ψ, i.e., the preferred sign in both ϕ and ψ. We assess the energetics of "extended" to "folded" transition as described under Materials and Methods. The results are in Table 3. Accessibility-based hydrophobic energy (E_{Bu}) and Lennard-Jones energy (E_{LJ}) tend to fold the extended structures irrespective of stereochemistry (Table 3). The remaining terms concern the peptide dipoles interacting mutually and with solvent. We compute the latter as electrostatic-solvation energy (E_{ES}) with DelPhi[49] using water as reference probe, and the former as Coulomb energy, which we resolve sequentially and spatially as hydrogen-bond (C_{Hb}) and non-hydrogen-bond terms, the latter sequentially local (C_{Lo}) and nonlocal (C_{Nl}). From the data in Table 3, we find hydrogen bond (C_{Hb}) and Coulomb nonlocal energies (C_{Nl}) tending to fold the extended structures irrespective of stereochemistry (Table 3). In contrast, we find electrostatic-solvation (E_{ES}) and Coulomb local energies (C_{Lo}) tending to "fold" or "unfold" the structures based on stereochemistry (Table 3). Thus, the terms under our consideration are in harmony in alternating-L,D peptide but conflicted in poly-L peptide, making the latter frustrated due to stereochemical reasons.

The frustration may mediate in the control of folding in poly-L peptide. As solvent, water promotes solvophobic aggregation (E_{Bu}), solvates peptides (E_{ES}), competes with hydrogen bonds (C_{Hb}), and screens electrostatics (C_{Nl} and C_{Lo}). Dependent on solvent or susceptible to its effect, E_{Bu}, C_{Hb}, E_{ES}, C_{Nl}, and C_{Lo} may all contribute in folding free energy of poly-L peptide. However, being isomer specific, E_{ES} and C_{Lo} may mediate in the selection between the secondary structures locally folded—turns, loops, and helical structures obligated to having at least some α-type ϕ,ψs—and extended—β-structure having no α-type ϕ,ψs. Considering the magnitudes, C_{Lo} could be the stronger constraint than E_{ES} in mediating the selection between secondary structures. Indeed, we find poly-L peptide having the specificity in secondary structure (% hydrogen bonds SR, MR, and LR) (Table 2) and the effective strength in hydrogen bonds ($\Delta E_{tot}/N_{Hb} < -13$ kJ/mol/hydrogen bond, Table 3) determined primarily with C_{Lo} via its solvent-mediated screening. Indeed, folded by this term and thus inert to dielectric, the alternating-L,D peptide is in the specificity of secondary structure and the effective strength of hydrogen bonds (> −43 kJ/mol/hydrogen bond, Table 3) inert to solvent. Thus, homochiral stereochemistry is critical to solvent-mediated control of folding in poly-L peptide primarily due to C_{Lo}. C_{Lo} is crucial to protein folding in its sequence-mediated control according to the electrostatic-screening hypothesis of Avbelj.[50]

Discussion

We reasoned that proteins manifest in their folding a critical balance between the forces in mutual opposition that are tunable with solvent and side chains. We further reasoned that the forces known to favor folding or unfolding, most notably, hydrogen bonds in main chain,[5] solvophobic aggregates of side chains,[9,10] polymer entropy of unfolded-polypeptide structure,[13] and solvation of peptide dipoles,[12] do not provide a logical basis for understanding folding in its solvent- and sequence-mediated control. We suspected the electrostatics of poly-L-linked peptides to be involved, as was implied in a puzzling observation of Flory reported in 1967.[51,52] Flory found that, when mutated to alternating-L,D structure, the random coil in the poly-L structure collapsed 10-fold, diminishing to a characteristic ratio apparently physically unrealistic. Addressing the puzzle, we found our suspicion confirmed;[21,22] the electrostatics of peptide-chain structure turned out to be critical due to its conflict with hydrogen-bond-mediated folding specifically when the structure was poly-L. In the present study, we found further proof for our stereochemical hypothesis. The antagonistic forces of protein folding providing the basis for its control are the electrostatics and hydrogen bonds of peptide dipoles, and specifically when the dipoles are linked homochirally. We will now argue that the proposed conflict, stereochemical in its basis, will explain not only some of the apparent puzzles of protein folding but also its joint control with solvent and side chains.

The methylene bridges of protein main-chain structure are always L in configuration when carrying side chains. Thus, the

TABLE 2: Modeling Folding−Unfolding Equilibria with Molecular Dynamics (Density and Specificity of Microstates of Isomeric Peptides under Specific Solvents)

			no. of microstates		hydrogen bonds in E_{50}				ϕ, ψ distribution in E_{50}			
stereochemistry	solvent[a]	no. of conformers	in E_{100}[b]	in E_{50}[b]	Av./Conf.	% SR[c]	% MR[c]	% LR[c]	% α[d]	% β[d]	% ϕ_F[d]	% ϕ_U[d]
					Ac-Ala_8-NMHe							
poly-L	m	23400	390	11	0.13	75	17	8	4.69	95.50	97.84	2.16
	w	21600	859	52	0.97	26	49	25	20.74	66.23	83.12	16.88
Alt-L,D	m	23400	568	20	1.23	3	55	42	4.81	93.60	99.18	0.82
	w	19700	674	19	1.25	6	42	52	8.66	87.81	97.77	2.23
					Ac-Lys-Ala_3-Lys-Ala_3-Lys-NH_2							
poly-L	m	25000	396	10	0.41	94	6	0	4.0	95.0	98.7	1.3
	w	25000	567	18	2.87	28	39	32	33.0	58.0	95.5	4.5
Alt-L,D	m	25000	354	5	3.84	7	37	55	3.0	93.0	99.3	0.71
	w	25000	173	5	3.27	7	27	65	7.0	89.0	97.9	2.1

[a] m = methanol, w = water. [b] E_{100} = 100% ensemble, E_{50} = 50% ensemble lower in free energy. [c] SR = short ranged (i-i ± 2), MR = medium ranged (i-i ± 3 + i-i ± 4), LR = long ranged (i-i ± 5 + i-i ± ≥6); [d] Basin definitions are as follows. α: $^{L/D}\phi = \mp 20$ to ∓ 100, $^{L/D}\psi = \mp 20$ to ∓ 80. β: $^{L/D}\phi = \mp 30$ to ∓ 170, $^{L/D}\psi = \pm 80$ to ± 180. ϕ_F: $^{L}\phi = 0$ to -180, $^{D}\phi = 0$ to 180. ϕ_U: $^{L}\phi = 0$ to 180, $^{D}\phi = 0$ to -180.

TABLE 3: Changes in Energy (ΔE or ΔC), Number of Hydrogen Bonds (ΔN_{Hb}), and Energy per Hydrogen Bond ($\Delta E_{tot}/\Delta N_{Hb}$) on the Folding of Solvent-Specific (w/m) Microstates (M_1) or Ideal Folds Fully Folded (IF_F) of Ala Peptide (Ac-Ala_8-NHMe) from the Ideal Folds Fully Extended (IF_E) (All Values Are in kJ/mol)[a]

	ΔE_{LJ}	ΔE_{Bu}	ΔE_{Es}	ΔC_{Hb}	ΔC_{Lo}	ΔC_{Nl}	ΔE_{Tot}	ΔN_{Hb}	$\Delta E_{Tot}/\Delta N_{Hb}$
					Poly-L				
$IF_E \rightarrow M_1$(m)	6.5	−0.4	11.2	0.0	−13.4	5.5	12.2	0	0.0
$IF_E \rightarrow M_1$(w)	−35.5	−17.3	19.9	−15.3	41.2	−24.8	4.6	2	2.3
$IF_E \rightarrow IF_F$	−64.6	−11.9	35.5	−114.0	122.0	−45.0	−76.0	6	−12.7
					Alternating-L,D				
$IF_E \rightarrow M_1$(m)	−8.4	−1.3	−1.4	−18.0	−129.5	−15.1	−154.0	2	−77.0
$IF_E \rightarrow M_1$(w)	−40.2	−9.4	4.0	−36.7	−128.3	4.8	−170.0	4	−42.5
$IF_E \rightarrow IF_F$	22.8	−5.7	−70.4	−34.2	−134.0	−3.8	−225.3	3	−75.1

[a] IF_E: ideal fold, fully extended. IF_F: ideal fold, fully folded. M_1(w/m): microstate 1 in water/methanol. E_{LJ}: Lennard-Jones energy. E_{Bu}: accessibility-based hydrophobic burial energy. E_{Es}: Electrostatic-solvation energy against water as test probe. C_{Hb}: Coulomb energy over the peptide groups mutually hydrogen bonded. C_{Lo}: Coulomb local energy over the peptide groups within a residue and its immediate sequence neighbors; C_{Nl}: Coulomb non-local energy = $C_{Tot} - (C_{Lo} + C_{Hb})$, with C_{Tot} being the Coulomb energy over all peptide groups. $E_{Tot} = E_{LJ} + E_{Bu} + E_{ES} + C_{Tot}$. N_{Hb}: number of hydrogen bonds.

polymer structure is always poly-L except in the positions occupied with unsubstituted glycine being achiral in structure. The consequence of poly-L stereochemistry to the folds of protein structure has been clear over decades. The features relevant for this study, illustrated in Figure 1, are the preferred sign in ϕ,[16,17] the topological options of hydrogen bonds for α-helix and β-sheet secondary structures (the options are in correspondence of the choice of sign in ψ),[2,3] and the regularity of peptide-dipole vectors mutually parallel (in α-helix) or antiparallel (in β-strand). The specific notion we are introducing here is that the coherence of peptide-dipoles due to the stereochemical regularity of poly-L structure may endow proteins with not only their characteristic cooperativity in folding but also the basis for its control. Specifically critical could be that the hydrogen bonds and the electrostatics of peptide dipoles are in mutual conflict when the structure is poly-L, being in harmony when the structure is alternating-L,D, as can be noted in Figure 1. The likely role of homochiral stereochemistry in causing protein structure to become energetically frustrated, while implied in Flory's puzzle[47,51,52] and in Avbelj's electrostatic-screening model,[50] and elaborated by Ramakrishnan et al.,[21,22] has been manifest clearly in a number of the recent reports which we shall now summarize.

Short peptides are proven solidly to adopt in water a semiextended structure PPII in conformation.[12,14,19,53,54] The underlying reason, dissected in a rigorous quantum mechanics-assisted theoretical study,[12,26–30] is the intrinsic preference of the poly-L peptide backbone for extended-β structure (favored by C_{Lo}) against the effect of solvation in causing ϕ,ψs to relax from β to PPII (implicating a role for E_{Es}) conformation. That the preference in the structures to be fully or semiextended could be stereoelectronic in its basis was indicated in the calculations according to which the same peptides were unstable as helical folds;[27] presumably, the repulsive electrostatics of the dipoles mutually parallel due to poly-L structure (unfavorable C_{Lo}) outweighs the favorable effect of hydrogen bonds between them (favorable C_{Hb}). The peptide chains shorter than about 10 residues, indeed, do not form a stable α-helix; the 3_{10}-helix is relatively preferred,[27] apparently because fewer dipoles are in unfavorable electrostatics compared with the dipoles that are mutually hydrogen bonded. The enthalpy of the hydrogen bond in the helical fold measured with calorimetry[7,55] and confirmed in both empirical[7] and quantum-mechanical calculations[26] apparently is a residual of the unfavorable electrostatics (C_{Lo}) and the favorable hydrogen bonding (C_{Hb}) in the helix homochiral in structure, as is proven in the calculations in our reports, the present and previous.[22] As might be expected based on the calculations, short peptides of amyloidogenic structure are in the gas phase β-hairpins,[31] apparently favored by the peptide dipoles of poly-L structure being antiparallel (favorable C_{Lo}) and yet mutually hydrogen bonded (favorable C_{Hb}). The oligopeptides similar in length are either β-hairpins or PPII helices in solution, as noted in this study, and the screening of electrostatics of poly-L-chain structure is a consideration for the solvent role. With increasing chain length, the α-helix becomes the preferred fold,[12,18] presumably lowered in free energy relative to the hairpin because the unfavorable electrostatics of the dipoles of poly-L structure finds compensation in the adequate number of

the dipoles that can hydrogen bond helically and thus benefit from the cooperativity of the helical structure.[28,29] Thus, in the solvent-mediated control of folding in poly-L peptide, the screening of the electrostatics of the homochirally joined dipoles (C_{Lo}) may serve as the critical principle. The effect is critical to side-chain-mediated control of protein folding according to the electrostatics-screening model of Avbelj.[50]

Avbelj, Baldwin, and co-workers have reported extensive studies of the solvation of the poly-L peptide chain under the influence of the electrostatics of peptide dipoles as calculated with DelPhi.[37–43] The results, while emphasizing the nonadditivity of solvation energy,[43] reflecting perhaps the fundamentally long-ranged nature of electrostatics, have implicated for side-chain structures steric interference in the access of solvent a critical role. The studies have found that the conformation-dependent NMR chemical shifts in the polypeptide backbone,[41] propensities in specific side-chain structures for α-helix[40] and β-sheet secondary structures,[39] and near-neighbor effects of specific side-chain structures on $^3J_{NH-C\alpha H}$ values in short peptides[37] are all correlated with side-chain effects in solvent access in the peptide chain. The steric bulk in side chains closer to the main chain, it has been argued, is the critical effect determining the degree of access the peptide dipoles have to solvent water.[37] Taken together, the results could be interpreted as implying that the "solvation" of specific folds of poly-L structure, modulated by side-chain sterics, could be critical to the control of protein folding. However, a quantum mechanics-assisted calculation has established that the differential of solvation energy in "helical" and "random" conformational folds of a poly-L-alanine peptide is much too small to implicate for "solvation" a role critical for the control of folding−unfolding equilibrium.[12] Possibly, the modulation of solvation by side-chain sterics, reflected in the reported DelPhi calculations,[37–43] manifests for water a role in protein folding not as "solvent" for dipoles but as "dielectric" for their electrostatics.

Conclusion

Homochiral stereochemistry being ubiquitous to protein structure was examined for possible relevance to the energetics of protein folding. Combined experimental and computational studies have established that suitable oligoalanines are two-state in folding−unfolding equilibria when the structure is poly-L but not when it is alternating-L,D. By calculating the energetics associated with the equilibria, we have found that the electrostatics of peptide dipoles mediates in the control of folding because of its conflict with mutual hydrogen bonds of the dipoles due to their linkages being poly-L. Thus, homochiral structure may provide in protein folding the basis for its control with screening of peptide-chain electrostatics being frustrated due to stereochemical reasons.

Materials and Methods

Experimental Studies. Amino acids with suitable protecting groups were purchased from Novobiochem (Switzerland). Poly-L and alternating-L,D isomers of Ac-^{L}Lys-$^{L/D}$Ala-^{L}Ala-$^{L/D}$Ala-^{L}Lys-$^{L/D}$Ala-^{L}Ala-$^{L/D}$Ala-^{L}Lys-NH_2 were synthesized manually using standard solid phase Fmoc chemistry[56] with the TBTU/HOBT or HOBT/DIC method on Rink Amide AM resin at a 0.74 mM/g scale. The resin was deblocked at the N-terminus by 30% piperidine−DMF with continuous shaking for 0.5 h. Kaiser test was used for monitoring the completion of coupling or deblocking of amino groups. The N-terminus was capped by acetylation (−$NHCOCH_3$) with Ac_2O:DIPEA:DMF in the ratio 1:2:20 over 5 h. The final peptide was obtained as a primary amide group (−$CONH_2$) at the C-terminus. Simultaneous cleavage of the peptide from the resin bed and deprotection of side chains was achieved with reagents-K [TFA:H_2O:phenol:thioanisole:EDT in a ratio of 82.5:5:5:5:2.5] over 1.5−4.0 h at room temperature with continuous shaking. After removal of TFA by passing N_2 gas, the peptide was precipitated with cold diethyl ether (anhydrous), and lyophilized. Peptide purity was checked by HPLC over an RP-18 silica column (5 μm, 4 mm × 250 mm; Merck) using CAN/H_2O/0.1%TFA/0−100% gradient. The identity of the desired structure was confirmed by MALDI-TOF mass spectrometry (M_{calc}, 869 Da; M_{H+}, 870 Da; M_{Na+}, 892 Da; M_{K+}, 908 Da, Supporting Information, Figure S2) and ^{1}H NMR analysis.

^{1}H NMR spectra of poly-L and alternating-L,D isomers of the peptide Ac-^{L}Lys-$^{L/D}$Ala-^{L}Ala-$^{L/D}$Ala-^{L}Lys-$^{L/D}$Ala-^{L}Ala-$^{L/D}$Ala-^{L}Lys-NH_2 were recorded in citrate buffer (pH ∼3) at 2.5 and 0.25 mM concentration at 298 K, and VT spectra were recorded at 2.5 mM between 20 and 50 at 10 °C intervals on an 800 MHz Bruker NMR instrument equipped with a cryoprobe (Supporting Information, Figure S3-5). CD measurements were made at 298 K in the far UV region on a JASCO J-810 CD spectropolarimeter equipped with a peltier controller [0.2 cm quartz cell, 700 μL volume, scanning rate of 100 nm/min, time constant 1.0 s] over an average of five accumulations with a step resolution of 0.5 nm, and bandwidth of 2 nm. The thermal melt of the peptide (∼40 μM) was recorded with 3 min thermal equilibration at each temperature. No significant change in molar ellipticity was observed in the 40−100 μM concentration range, suggesting the absence of aggregation (Supporting Information, Figure S6). In order to avoid discrepancies of peptide concentration, all experiments were performed from a single stock solution of the peptide. DSC experiments were performed on a METTLER Toledo DSC-822 calorimeter (Microcal, Inc.). The peptide in water at ∼2 mg/50 μL concentration was rescanned after the first run to check for possible reversibility of transitions in the range 15−90 °C scanning at the rate 5 °C/min (Figure 3).

Computational Studies. Molecular dynamics was performed with the gromos-96 43A1 force field in the GROMACS package[57,58] in a periodic box of explicit solvent under NVT, viz., constant number of particles, volume, and temperature.[21] The nonbonded list cutoff was 1.4 nm with shift at 0.8 nm. The integration step was 2 fs. Initial velocities were from Maxwellian distribution. The temperature was coupled to an external bath with a relaxation time constant of 0.1 ps. Bond lengths were constrained with SHAKE[59] to a geometric accuracy of 10^{-4}. In simulations involving poly-Ala (Ac-Lys-Ala_3-Lys-Ala_3-Lys-NH_2), three Cl^- counterions were added as a replacement for solvent molecules. The electrostatics was in this case treated by the particle mesh Ewald (PME) method[60,61] implementing a Coulomb cutoff of 1.4 nm, a Fourier spacing of 0.12 nm, and an interpolation order of 4. Poly-L Ac-Ala_8-NHMe was modeled as the ideal-fold fully extended (IF_E) with $\phi = -120°$, $\psi = 120°$ and fully folded (IF_F) with $\phi = -57°$, $\psi = -47°$. Alternating-L,D isomer was modeled as the ideal-fold fully extended (IF_E) $\phi^{L/D} = \mp 57°$, $\psi^{L/D} = \mp 47°$ and fully folded with the ϕ,ψs in correspondence of the residues 3−10 of the Gramicidin-A β-helix (PDB: 1GRM);[62] we represent these values notionally as $\phi^{L/D} \sim \pm 120°$, $\psi^{L/D} \sim \pm 120°$. The peptide constrained to the center of the periodic cubic box of edge length 4.5 nm was soaked with the SPC water model[63] or with the methanol model as furnished in the gromos-96 force field, which was added to 1 atm density at 298 K. The solute was energy minimized, then the solvent was energy minimized after restraining solute, and then both were energy minimized after

removing restraint. MD was initialized, 3 ns was exempted as the pre-equilibration period, and the trajectory was sampled at the 10 ps interval.

Peptide conformers were clustered in Cartesian space[64] to ≤0.15 nm rms cutoff, giving microstates diminishing in population. Radius of gyration (R_g) was calculated using the g_gyrate utility in GROMACS package.[57,58] Hydrogen bond was enumerated to 0.35 nm distance (N−O) and 30° angle (H−N−O) cutoff. Specific definitions for hydrogen bonds are SR = short ranged (i to i ± 2), MR = medium ranged (i to i ± 3 and i to i ± 4), and LR = long ranged (i to i ± 5 and i to i ± ≥6) according to the sequential separation between the donor and acceptor residues of the hydrogen bond (Supporting Information, Figure S1). Specific ϕ,ψ basin definitions are $^{L/D}\alpha$: $^{L/D}\phi = \mp 20$ to ∓ 100, $^{L/D}\psi = \mp 20$ to ∓ 80; $^{L/D}\beta$: $^{L/D}\phi = \mp 30$ to ∓ 170, $^{L/D}\psi = \pm 80$ to ± 180; ϕ_{SF}: $^{L}\phi = 0$ to -180, $^{D}\phi = 0$ to 180; ϕ_{SU}: $^{L}\phi = 0$ to 180, $^{D}\phi = 0$ to -180, as given in Figure 1.

Lennard-Jones energy (E_{LJ}) was calculated with the parameters for 12−6 equation drawn from gromos-96 using the standard program g_energy in GROMACS.[57,58] Coulomb energy was calculated under dielectric constant (ε) one with the charge parameters drawn from gromos-96. The Coulomb energy over all peptide groups gave C_{Tot}, over the peptide groups within a residue and in its immediate sequence neighbors, as defined in Figure S1 of the Supporting Information, gave C_{Lo}. The Coulomb energy over the peptide groups satisfying the distance cutoff ≤0.35 nm and angle cutoff ≤30° gave the Coulomb hydrogen bond energy C_{Hb}. C_{Nl} was calculated by the relation $C_{Nl} = C_{Tot} - (C_{Lo} + C_{Hb})$.

Solvent-accessible surface area (ASA) was calculated with NACCESS[65] using the probe radius 1.4 Å. Hydrophobic-burial energy (E_{Hb}) was calculated over Cα and Cβ atoms as $E_{Bu} = k_h(ASA_{PPII} - ASA_{Fold})$, with ASA_{PPII} and ASA_{Fold} being the solvent accessible surface area in the standard-PPII conformer and the fold of interest. The value for proportionality coefficient k_h was taken as 13.0 kJ mol^{-1} nm^{-2}.[66]

Electrostatic-solvation free energy (E_{SE}) was calculated for specific structures or for the microstates in their central members, after being fitted with hydrogens using GROMACS.[57,58] Finite-difference solution of the Poisson−Boltzmann equation was implemented with DelPhi[49] using water as a universal reference probe for all of the calculations reported. The internal and external dielectric values were, respectively, 4.0 and 80.0; the probe radius was 1.4 Å; the grid size was 65 with scale 1.0; the convergence threshold value was maxc = 0.0001.

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Supporting Information Available: Figures showing a schematic representation of hydrogen bonds of polypeptide structure, MALDI-MS spectra and HPLC traces, ^{1}H-NMR spectra, temperature dependence of amide-NH chemical shifts, chemical shifts of amide-NH resonance, CD spectra, molecular dynamics, residue-by-residue occupancy, and the central members of the 10 most populous microstates of octa-peptide and nona-peptide. Tables showing energetics of intrachain and chain−solvent interaction and population statistics, hydrogen bond statistics, and specific intrachain and chain−solvent interaction terms. This material is available free of charge via the Internet at http://pubs.acs.org.

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