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The chicken-egg scenario of protein folding revisited

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Abstract **What is the first step in protein folding – hydrophobic collapse (compaction) or secondary structure formation? It is still not clear if the major driving force in protein folding is hydrogen bonding or hydrophobic interactions or both. We analyzed data on the conformational characteristics of 41 globular proteins in native and partially folded conformational states. Our analysis shows that a good correlation exists between relative decrease in hydrodynamic volume and increase in secondary structure content. No compact equilibrium intermediates lacking secondary structure, or highly ordered non-compact species, were found. This correlation provides experimental support for the hypothesis that hydrophobic collapse occurs simultaneously with formation of secondary structure in the early stages of the protein folding.** © **2002 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.**

Key words: Protein folding; Hydrophobic collapse; Secondary structure formation; Partially folded intermediate

1. Introduction

Understanding the initial events in protein folding is an important prerequisite to solving the problem of protein self-organization. Long-standing controversial questions include the nature of the first step in this process – hydrophobic collapse (compaction) or secondary structure formation – and the nature of the major driving force for protein folding – hydrogen bonding or hydrophobic interactions. As early as 1937 it was suggested that protein folding is directed mostly by the formation of intramolecular hydrogen bonds [1]. Based on this hypothesis, the secondary structure framework model, postulating the stepwise formation of a rigid protein structure, was elaborated. According to this model, the polypeptide chain undergoes local folding to native-like elements of secondary structure, which direct subsequent folding through diffusion/collision processes [2,3]. On the other hand, it has been noted that the hydrophobic attraction could be the dominant force governing protein folding due to the fact that hydrogen bonding to the solvent molecules would strongly favor the unfolded state [4]. This hypothesis gave rise to the hydrophobic collapse model, according to which strong hydrophobic attractions should first lead to non-specific compaction of the polypeptide chain into a structure-less globule. This step significantly reduces the conformational space and consequently facilitates the formation of secondary and tertiary structure [5–8]. Discussions of these fundamentally different competing models have generated considerable controversy over the years. To some extent this is reminiscent of the debates concerning the old philosophical paradigm, which was first – the chicken or the egg?

Equilibrium partially folded conformations are usually considered stable counterparts of kinetic intermediates transiently populated during protein refolding kinetics [9]. Many globular proteins have been shown to exist in multiple stable conformations, e.g. the native, molten globule, pre-molten globule, and unfolded states [10–13]. In an attempt to elucidate the mechanism involved in the initial events of protein folding, the literature data on the conformational characteristics of several globular proteins in native and partially folded conformational states have been analyzed. A good correlation between relative decrease in hydrodynamic volume and increase in secondary structure content has been established.

2. Materials and methods

Literature data on the equilibrium unfolding of globular proteins have been analyzed to find a set of proteins for which secondary structure and Stokes radii, R_S, of native, unfolded and different partially folded intermediates have been evaluated by far-UV circular dichroism (CD) and hydrodynamic methods, respectively (see Table 1). The degree of compactness and the relative amounts of ordered secondary structure have been calculated for different conformational states. The degree of compactness was calculated as the decrease in hydrodynamic volume of the given conformation relative to the volume of the unfolded conformation, $(R_S^U/R_S)^3$. The relative amount of ordered secondary structure was calculated for different conformational states from their far-UV CD spectra as the increase in negative ellipticity at 222 nm, $[\theta]_{222}$, relative to that of the unfolded conformation, $[\theta]_{222}/[\theta]_{222}^U$.

3. Results and discussion

Our analysis of literature data on the equilibrium unfolding of globular proteins revealed a set of 41 proteins for which secondary structure and Stokes radii, R_S, of native, unfolded and different partially folded intermediates have been evaluated (see Table 1). Using this set of proteins, the correlation between the degree of compactness and corresponding secondary structure content was analyzed. The results of this analysis are presented in Fig. 1 as $(R_S^U/R_S)^3$ (relative compactness) vs. $[\theta]_{222}/[\theta]_{222}^U$ (relative content of ordered secondary structure). In this plot open and closed circles correspond to the data for native globular proteins and their partially folded intermediates, respectively. Data for both classes of conformations (native states and various intermediate states) may be described

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Table 1
Structural characteristics of the analyzed proteins in different conformational states

Protein (MW)	$[\theta]_{222}$	$[\theta]_{222}/[\theta]^{U}_{222}$	R_S	$(R^{U}_S/R_S)^3$	Reference
Albebetin, de novo (7 760)	U: −3 100		U: 24.1		[39]
	MG: –9 310	3.00	MG: 17.2	2.75	
Albeferon, de novo (8 640)	U: −3 000		U: 25.3		[39]
	N′: −9 750	3.25	N′: 16.5	3.61	
Stefin B (11 140)	U: −1 350		U: 29.9		
	PMG: −2 850	2.11	PMG: 22.4	2.37	[40]
	MG: −7 550	5.59	MG: 17.5	4.98	
	N: −6 350	4.70	N: 17.3	5.16	
Cytochrome *c* (11 702)	U: −1 250		U: 34		
	MG: −9 600	5.65	MG: 20	4.91	[41]
	N: −17 000	7.94	N: 17	8.00	
Prothymosin α, human (12 072)	U: −2 000		U: 31.4		
	PMG: −4 760	2.38	PMG: 24.9	2.00	[42]
	PMG: −4 100	2.56	PMG: 23.4	2.42	
−6–102 fragment of SNase R (12 099)	U: −1 850		U: 31.2		
	PMG: −4 000	2.16	PMG: 23.6	2.31	[43]
α-Lactalbumin (human) (14 078)	U: −4 000		U: 25.8		
	MG: −9 321	2.33	MG: 20.2	2.08	[44]
	N: −10 640	2.96	N: 18.0	2.94	
α-Synuclein, human (14 460)	U: −1 300		U: 34.3		[45]
	PMG: −2 500	1.92	PMG: 28.0	1.84	
Intestinal fatty acid binding protein (15 076) and its	U: −1 000		U: 36.4		
1–128 fragments (14 705)	U_{1-128}: −1 000		U_{1-128}: 36.1		[46]
	N_{mut}: −5 600	5.60	N_{mut}: 20.6	5.51	
	N_{wt}: −6 200	6.20	N_{wt}: 20.0	6.03	
	N_{1-128}: −6 200	6.20	N_{1-128}: 19.7	6.15	
			PMG_{1-128}: 28.0	1.93	
			28.0	1.98	
			PMG_{mut}: 29.0	1.98	
			PMG_{wt}: 29.0		
Tumor suppressor protein P16 and its mutants	U: −1.6		U: 37.1		
(16 532)	N_{H98P}: −3.9	2.44	N_{H98P}: 30.3	1.85	[47]
	N_{R24P}: −4.5	2.81	N_{R24P}: 28.9	2.12	
	N_{P81L}: −6.8	4.25	N_{P81L}: 23.6	2.71	
	N_{wt}: −7.0	4.38	N_{wt}: 22.2	3.88	
	N_{D74N}: −7.2	4.50	N_{D74N}: 22.2	4.67	
	N_{D84N}: −8.9	5.57	N_{D84N}: 20.0	6.38	
SNase (16 811)	U: −3 000		U: 37.2		
	A_1: −7 800	2.60	A_1: 29.2	2.06	[16]
	A_2: −9 830	3.06	A_2: 27.9	2.37	
	A_3: −13 884	4.60	A_3: 23.2	4.12	
	N: −14 120	5.69	N: 19.7	6.23	
SNase F34W/W140F mutant (16 811)	U: −2 500		U: 37.2		
	Mut: −8 000	3.20	Mut: 25.0	3.29	[48]
	Wt: −12 000	4.80	Wt: 21.5	5.18	
Apo-myoglobin (16 951)	U: −5 000		U: 34.0		
	A_1: −12 000	2.40	A_1: 27.2	1.95	[49]
	A_2: −15 000	3.00	A_2: 25.9	2.26	
	A_3: −19 000	3.80	A_3: 22.4	3.50	
	N: −22 000	5.65	N: 18.7	6.01	
Dihydrofolate reductase (17 578)	U: −500		U: 37.6		
	MG: −2 800	5.60	MG: 22.5	4.67	[50]
	N: −3 200	6.40	N: 20.5	6.17	
Equine β-lactoglobulin (18 500)	U: −1 450		U: 37.0		
	MG: −5 000	3.45	MG: 24.0	3.66	[51]
	N: −6 950	4.79	N: 22.0	4.76	
Apoflavodoxin (18 832) and its 1–149 fragment	U_{frag}: −1 550		U_{frag}: 37.7		
(16 468)					
	MG_{frag}: −5 100	3.29	MG_{frag}: 24.3	3.73	[52]
	MG_{frag}: −5 900	3.81	MG_{frag}: 23.8	3.98	
	N: −8 775		N: 22.9		
Human nucleoside diphosphate kinase A (18 900)	U: −1 100		U: 40.8		
	MG: −5 320	4.84	MG: 25.0	4.35	[53]
	N: −7 580	6.88	N: 21.1	7.22	
Nereis sarcoplasmic calcium binding protein (19 485)	U: −1 750		U: 40.5		
	MG: −9 000	5.14	MG: 24.2	4.69	[54]
	N: −11 000	6.28	N: 21.5	6.68	
Adenylate kinase (21 658)	U: −1100		U: 42.1		
	PMG: −2 300	2.09	PMG: 30.3	2.68	[55]
	MG: −6 500	5.91	MG: 24.3	5.20	
	N: −8 200	7.45	N: 21.9	7.10	

Table 1 (*continued*)

Protein (MW)	$[\theta]_{222}$	$[\theta]_{222}/[\theta]^{U}_{222}$	R_S	$(R^{U}_S/R_S)^3$	Reference
Bovine growth hormone (21 745)	U: −2 000		U: 37.0		
	MG: −7 000	3.50	MG: 26.0	2.88	[56]
	N: −16 000	8.00	N: 18.0	8.69	
Ovine placental lactogen (21 780)	U: −3 168		U: 41.6		
	MG: −16 385	5.17	MG: 24.5	4.89	[57]
	N: −19 750	6.23	N: 22.4	6.40	
Peanut lectin monomer (25 000)	U: −0.125		U: 45.9		
	MG: −0.711	5.69	MG: 26.4	5.26	[58]
	N: −0.915	7.32	N: 23.4	7.54	
Trypsinogen (25 425)	U: −1 050		U: 38.1		
	PMG: −3 500	3.33	PMG: 24.1	3.95	[59]
	N: −8 100	7.71	N: 19.8	7.12	
Tryptophan synthase, α-subunit (28 724)	U: −1 590		U: 53.7		
	PMG: −7 200	4.53	PMG: 33.9	3.97	[60]
	N: −15 560	9.79	N: 24.2	10.93	
β-Lactamase, *Staphylococcus aureus* (28 794)	U: −1 250		U: 53.0		
	PMG: −6 000	4.80	PMG: 32.4	4.38	[10]
	MG: −9 750	7.80	MG: 26.7	7.82	
	N: −10 900	8.72	N: 25.7	8.77	
Bovine carbonic anhydrase B (28 800)	U: −600		U: 52.4		
	PMG: −3 000	5.00	PMG: 31.7	4.52	[12]
	MG: −4 700	7.83	MG: 26.4	7.82	
			N: 23.0		
RTEM β-lactamase (28 907)	U: −2 000		U: 45.0		[61]
	MG: −9 210	4.61	MG: 27.0	4.63	
	N: −12 100	6.05	N: 24.5	6.20	
β-Lactamase, *Bacillus cereus* (29 061)	U: −1 500		U: 51		
	MG: −11 000	7.31	MG: 26.5	7.12	[62]
	N: −13 000	8.67	N: 24	9.59	
Herpes simplex virus triplex protein VP23 (36 600)	U: −1 300		U: 48.4		[63]
	MG: −6 700	5.15	MG: 29.0	4.65	
Actin, human (42 050)	U: −1 250		U: 61.0		[64]
	N: −13 000	10.40	N: 27.5	10.91	
Ovalbumin (42 750)	U: −2 200		U: 61.13		[65]
	MG: −14 000	6.36	MG: 33.5	6.08	
	N: −14 000	6.36	N: 33.5	6.08	
Glutaminyl-tRNA synthetase (47 000)	U: −1 200		U: 60		[66]
	MG: −4 500	3.75	MG: 38	3.94	
	N: −5 000	4.17	N: 36	4.63	
Fetuin (48 000)	U: −750		U: 61.7		[67]
	MG: −3 620	4.83	MG: 38.5	4.11	
	N: −5 750	7.66	N: 36.3	8.35	
Interstitial collagenase (48 000)	U: −1 200		U: 60.6		[68]
	PMG: −4 950	4.13	PMG: 40.2	3.43	
	MG: −9 100	7.583	MG: 32.1	6.73	
	N: −12 500	10.42	N: 27.2	11.06	
Co-polymer (48 800)	U: −1250		U: 59.0		[69]
	PMG: −2 500	2.00	PMG: 47.2	1.95	
Bovine serum albumin (66 300)	U: −1 650		U: 81.8		[70]
	N: −24 180	14.65	N: 33.9	14.04	
α-Fetoprotein, human (66 478)	U: −1 450		U: 72.0		
	MG: −14 310	9.87	MG: 34.5	9.10	[70]
	N: −15 230	10.50	N: 32.4	10.97	
DnaK (68 983)	U: −1 800		U: 73.0		
	PMG: −4 500	2.50	PMG: 53.1	2.60	[14,15]
	MG: −16 000	8.89	MG: 36.3	8.13	
	N: −20 000	11.11	N: 32.5	11.33	
Creatine kinase (2*43 112)	U: −2 000		U: 61.2		[71,72]
	PMG: −4 600	2.30	PMG: 49.4	1.90	
	MG: −10 800	5.40	MG: 33.7	5.99	
Purple acid phosphatase (2*50 236)	U: −350		U_d: 101.6		[73]
			U_m: 71.7		
	N: −4 500	12.86	N: 41.7	14.46	
Acetylcholinesterase (2*60 673)	U: −1 100		U: 110.0		[74]
	MG: −6 500	5.91	MG: 59.0	6.48	
	MG: −9 000	8.19	MG: 52.0	9.47	
	N: –13 000	11.82	N: 49.0	11.31	

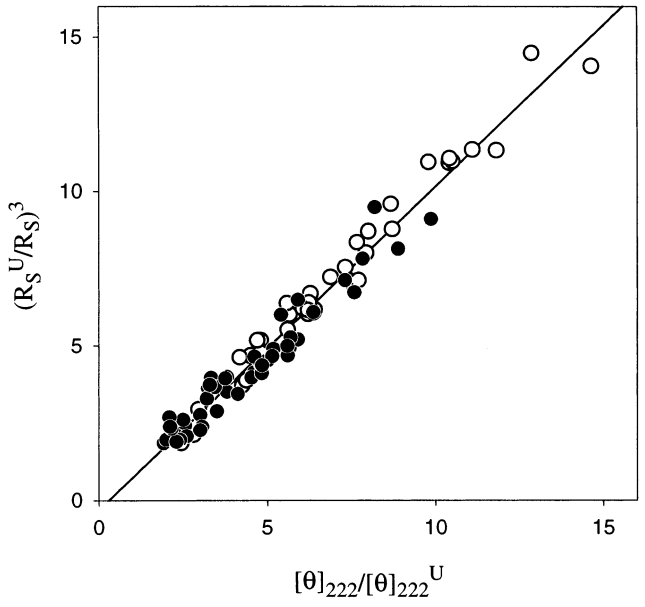

Fig. 1. Correlation between the degree of compactness (calculated for different conformational states as the decrease in hydrodynamic volume relative to the volume of the unfolded conformation) and amount of ordered secondary structure (calculated for different conformational states from their far-UV CD spectra as the increase in negative ellipticity at 222 nm, $[\theta]_{222}$, relative to that of the unfolded conformation). Open and closed symbols correspond to the data for native globular proteins and their partially folded intermediates, respectively.

by the expression (correlation coefficient $r^2 = 0.97$):

$$\left(\frac{R_S^U}{R_S}\right)^3 = (1.047 \pm 0.010)\cdot\frac{[\theta]_{222}}{[\theta]_{222}^U} - (0.31 \pm 0.12)$$

This means that the degree of compactness and the amount of ordered secondary structure are highly correlated. The idea of looking at the correlation between compactness and secondary structure content in proteins is not new. For example, such data have been provided for different conformational states of DnaK [14,15] and staphylococcal nuclease [16]. Furthermore, Goto and coworkers have performed extensive investigations on the dependence of the radius of gyration on helical content in apomyoglobin intermediates [17,18]. However, these prior works were focused on the description of different conformational states for a single protein, whereas the compilation in our study is more comprehensive and provides a broader picture. Thus, our analysis shows that there is no compact equilibrium intermediate lacking secondary structure nor any highly ordered but non-compact species among 41 proteins from the data set. In the context of the early stages of protein folding this suggests that hydrophobic collapse and the formation of secondary structure occur simultaneously, rather than representing two independent and sequential processes.

This conclusion is in a good agreement with recent developments in the characterization of structural features of unfolded proteins. It was long believed that proteins in strongly denaturing conditions, such as high concentrations of urea or guanidinium chloride, are random coils, i.e. ensemble of conformations involving only local interactions between residues. This follows from Flory's isolated pair model of the random coil, which states that the backbone conformation of every amino acid residue, as described by its ϕ and ψ pair of backbone angles, is independent of the conformation of the neighboring residues [19]. Interestingly, it has recently been shown that steric clashes among residues separated by three to six units eliminate a large number of backbone conformations in certain regions of the Ramachandran map [20]. However, it is well known in polymer physics that although local steric interactions can reduce the number of allowed conformations, they do not lead to any fundamental change in the exponential increase of accessible chain conformations as a function of chain length [21]. In agreement with this suggestion, extensive simulation studies by Derreumaux have shown that while dihedral biases increase the efficiency of conformational searches, they are not sufficient to fold proteins [22].

It is now becoming more and more evident that when a protein unfolds, not all of its structure is lost [23–36]. Moreover, it has been shown that long-range order and native-like spatial positioning and orientation of chain segments are present, even in concentrated solutions of strong denaturants. Thus, even the unfolded expanded polypeptide chain may have significant native-like topology under these conditions [36]. This means that the *unfolded protein is predisposed to adopt specific backbone conformations rather than to become a random coil*, as Flory postulated in his isolated pair hypothesis [19]. Therefore, favored backbone conformations already preexist in the denatured protein, effectively restricting the number of states accessible to the unfolded polypeptide chain [37]. Consequently, protein folding does not represent a random search for the favorable native structure throughout the enormously large conformational space. Instead, it can be considered a directed run within a rather narrow conformational corridor, on which the 'cementing' of the preexisting short- and long-range contacts occurs. In this view, the discussion of which happens first – compaction or secondary structure formation – is no longer relevant. The only acceptable scenario of the earliest stages of protein folding would be that the hydrophobic collapse happens simultaneously with the formation of secondary structure, a conclusion which is in complete agreement with the results of our analysis. This conclusion is supported by recent theoretical work of Kaya and Chan [38], who found that the experimentally observed thermodynamic and kinetic cooperativities in real proteins most likely arise from a cooperative interplay between local structural propensities and hydrophobic burial.

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