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Aponeocarzinostatin—A superior drug carrier exhibiting unusually high endurance against denaturants

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Abstract—The enediyne antitumor antibiotic chromoproteins are very potent in causing DNA damages. During the drug delivery time course, the stability of the carrier protein becomes an important concern. To simulate conceivably offensive environment in biological contexts, such as cell membrane, we studied structural endurance of aponeocarzinostatin against several denaturants by circular dichroism and nuclear magnetic resonance spectroscopy. For comparison, we also examined proteins known to be stable and similar in size to aponeocarzinostatin. The results highlight the unusual structural stability of aponeocarzinostatin against chemical denaturants, suggesting the potential of aponeocarzinostatin as an inherently superior carrier in drug delivery systems.

1. Introduction

The naturally occurring enediyne chromoproteins belong to a unique category with interesting combination, in which a potent but liable toxin is delivered to a target by a carrier protein. Without protection from the protein, the active toxin quickly degrades before the antitumor antibiotic could exert its effective action. How the carrier protein can manage its transferring task successfully is a fundamental issue to understand the drug delivery system. Conceivably, the conformational stability of the protein is the most important property that needs to be explored.

Neocarzinostatin (NCS) is the first enediyne antitumor antibiotic.[1] It consists of a nine-membered dienediyne chromophore (NCS-C)[2] bound non-covalently, but specifically and tightly, to an apoprotein (apoNCS).[3] NCS-C is very potent in causing DNA damage.[4,5] Although apoNCS by itself does not cause DNA damage, it contributes to the antimicrobial and anticancer activities of NCS by protecting labile NCS-C and also by delivering the same to the target DNA.[3]

ApoNCS is an 11 kDa, all-β-sheet protein of 113 amino acids (aa).[6–8] Figure 1 shows the simulated conformation of the protein in an aqueous environment.[9] NCS-C is bound in the cavity between the internal β-sheets of the larger domain and the smaller domain.[8] Nuclear magnetic resonance (NMR) studies suggest that apoNCS is able to bind with small molecules like naphthoate esters[10] or ethidium bromide.[11] Also, apoNCS improves the stability of potent DNA alkylating agents, nitrogen mustards.[12] An in vitro 'evolution' study[13] demonstrated that apoNCS can be engineered into a delivery vehicle for drugs that are completely unrelated to the natural enediyne chromophore.

During secretion of NCS by *Streptomyces carzinostaticus* and its transport to/across target cells, the important task of the carrier apoNCS is to protect and to stabilize NCS-C through specific binding interaction. Any form of unfolding of apoNCS can perturb such interaction and cause the protein to lose its protecting function. Consequently, conformational endurance of apoNCS

Abbreviations: NCS, neocarzinostatin; apoNCS, the apoprotein component of neocarzinostatin or aponeocarzinostatin; holoNCS, the chromoprotein of neocarzinostatin or holoneocarzinostatin; NCS-C, neocarzinostatin chromophore; CD, circular dichroism; mdeg, millidegree; NMR, nuclear magnetic resonance spectroscopy; 1D, one dimensional; 2D, two dimensional; HSQC, heteronuclear single quantum coherence; SDS, sodium *n*-dodecyl sulfate; GdnHCl, guanidine hydrochloride; TFE, 2,2,2-trifluoroethanol; IPA, isopropanol; *t*-BuOH, *tert*-butyl alcohol; aa, amino acid; C_m, concentration of the denaturant at which 50% of the protein molecules exist in the unfolded state; C_{min}, the lowest denaturant concentration required to induce any structural changes of a protein; CBP, calmodulin binding peptide.

Keywords: Aponeocarzinostatin; Antitumor antibiotic chromoprotein; Enediyne; Denaturants.

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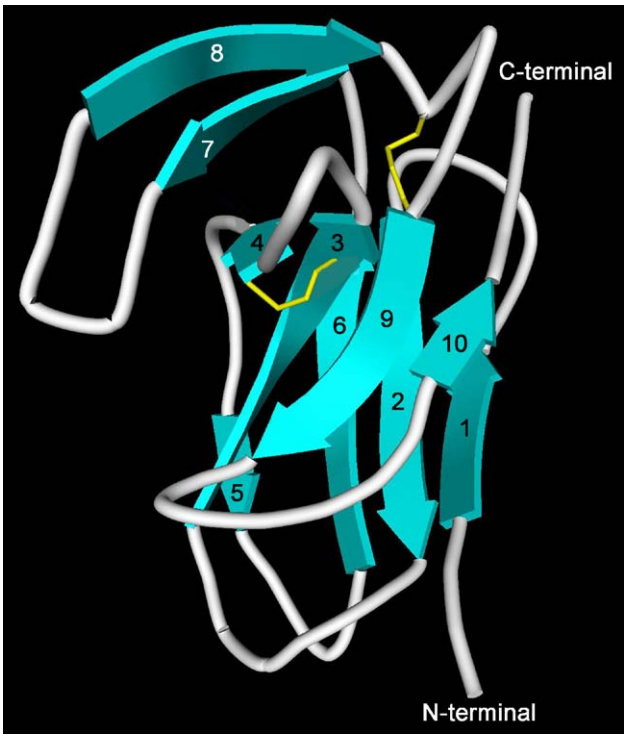

Figure 1. A schematic view of the apoNCS component in the NCS complex in an aqueous environment at neutral pH (modified[9] from Brookhaven pdb.1NCO.ent file). The protein backbone is shown in gray and cyan, and the two disulfide bonds are shown in yellow. All β-strands are numbered from N- to C-terminal.

against unfavorable environment at ambient temperature is vital during the drug transport time course. Thermal-induced transitions of apoNCS show that its T_m far exceeds ambient temperature in a broad range of pH 4–10.[14] Most bacteria including *S. carzinostaticus* cannot survive such high temperatures. With sufficiently high T_m, there is no need for the bacteria to concern the heat-induced instability of apoNCS. On the other hand, molecules from complex and offensive cellular environment are more likely to be a threat under physiological conditions at neutral pH. For instance, hydrophobic cell membrane, which is a barrier that NCS crosses while performing its cytotoxic action, has high potential to induce unfolding of proteins. In this context, the stability of apoNCS against chemical denaturants, which has a significant impact on the drug delivery system, needs to be explored systematically.

Effect of chemical denaturants on the conformation of NCS had not been thoroughly investigated. An early study on the primary structure of NCS[6] showed that NCS is extraordinarily resistant to reduction of disulfides, even in the presence of the highest possible concentrations of urea or guanidine hydrochloride (GdnHCl). Recently, we found that urea at the maximum concentration cannot induce the completely unfolded state of apoNCS at pH 7 and room temperature.[14] In the case of GdnHCl-induced unfolding, studies showed that complete denaturation of apoNCS can be achieved below 4 M GdnHCl at low temperatures.[15,16] Studies on NCS activity showed that some organic solvents stimulate DNA cleavage induced by holoNCS.[17,18] More recently, we studied the unfolding of apoNCS induced by 2,2,2-trifluoroethanol (TFE).[19] In brief, results of studies on the effect of chemical denaturants on the stability of NCS are scarce and not consistent.

In the present investigation, we aim to study the structural endurance of apoNCS against known chemical denaturants at room temperature. The biophysical characteristics of the unfolding of apoNCS induced by alcohols, acetonitrile, urea, GdnHCl, and sodium *n*-dodecyl sulfate (SDS) are examined by circular dichroism (CD) and NMR analyses. Comparative experiments are conducted side by side with cytochrome *c* and hen egg lysozyme, which are known to be stable and similar in size to apoNCS. We also compare the C_m values, the concentration of the denaturant at which 50% of the protein molecules exist in the unfolded state, with those of several other similar size proteins characterized elsewhere.

2. Results

2.1. Conformational transitions followed by CD

In general, the far-UV CD provides a good indication for the secondary structure of a protein. On the other hand, the near-UV CD signals are fingerprints of the compact and well-defined tertiary structure. The CD spectrum of the purified recombinant apoNCS is identical to that of the natural apoNCS [14] secreted by *S. carzinostaticus*, suggesting that the expressed recombinant apoNCS is folded as the native state. In aqueous solution, the near-UV CD of apoNCS shows a broad negative signal centered around 270–271 nm. The far-UV CD shows a large positive peak at 224 nm and a small negative peak at 211–212 nm, which is atypical for an all-β-sheet protein. We followed the denaturant-induced transitions of apoNCS at 25 °C by monitoring the CD signal at 224 and 270 nm for the secondary and tertiary structural changes, respectively.

2.1.1. Effect of organic solvents. We examined the effect of methanol, ethanol, isopropanol (IPA), *tert*-butyl alcohol (*t*-BuOH), and acetonitrile on the conformational stability of apoNCS at a wide range of concentrations at pH 5, at which apoNCS is known to be stable. The results show that both secondary and tertiary structural changes remain unchanged until very high concentration of these organic solvents (Fig. 2). In the case of methanol, the CD signal of apoNCS at 270 nm starts to decrease only above 65–70% of methanol. In all other solvents, there are no considerable changes in 270 nm signal up to 60% concentration. The results indicate that the aromatic side chains of apoNCS, such as Tyr32, Trp39, and Trp83, remain in a native-like steady and compact tertiary environment under these conditions. For the far-UV CD signals at 224 nm, no changes occur in these organic solvents up to concentration of 70% methanol, 65% ethanol, 60% IPA, or 60% *t*-BuOH. No significant changes in the secondary structure of apoNCS are observed in acetonitrile up to 80% concentration.

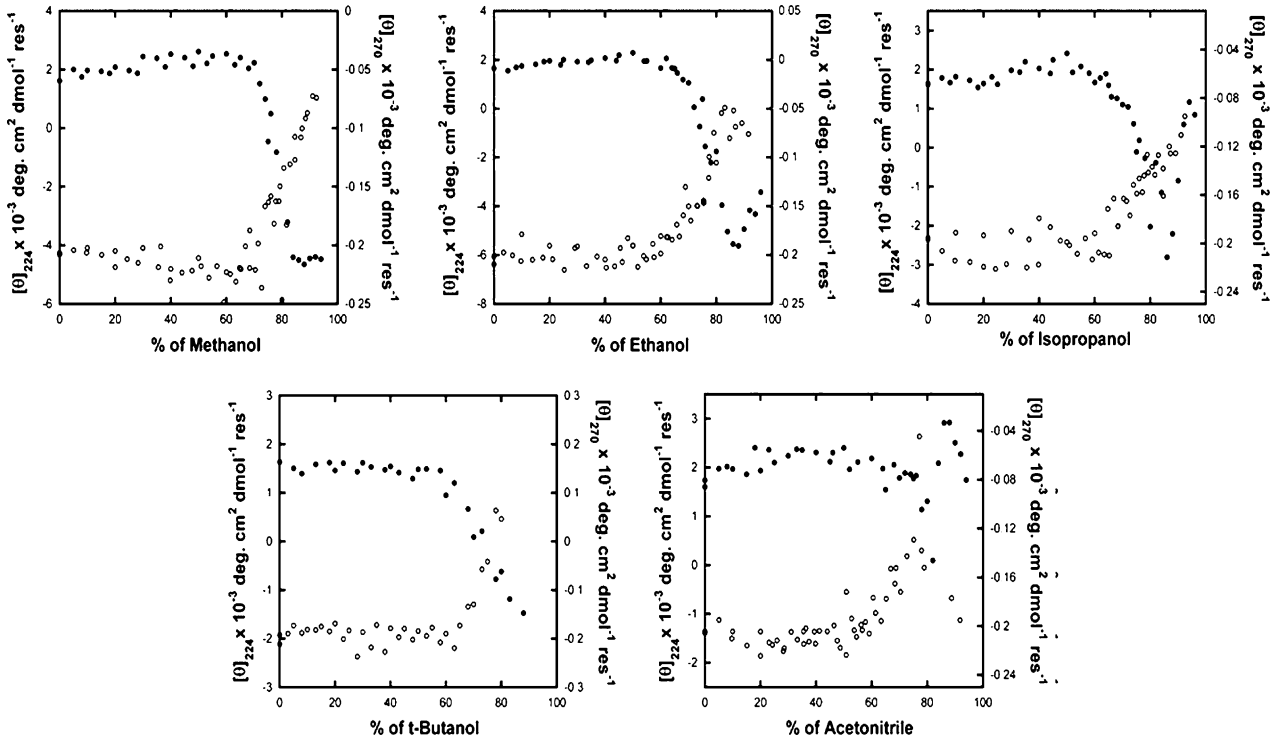

Figure 2. The observed changes in the mean residue ellipticity [θ] of apoNCS at 25 °C and pH 5 with respect to increasing concentration (v/v) of alcohols and organic solvents in far-UV CD at 224 nm (●) and near-UV CD at 270 nm (○).

The stability of a protein often depends on conditions. To examine whether the very high stability of apoNCS is an exceptional case, we also carried out comparative CD studies with a couple of proteins known to be stable under the same conditions. Since protein size is an important factor for stability, we selected hen egg lysozyme (129 aa) and horse heart cytochrome *c* (104 aa), whose sizes are comparable to that of apoNCS. Unfolding studies of cytochrome *c*, monitored by far-UV CD at its secondary structure signal at 208 nm, show that it is far less stable than apoNCS. Cytochrome *c* remains native-like state only up to concentration of 40% methanol, 25% ethanol, or 15% other solvents studied (i.e., IPA, *t*-BuOH, acetonitrile, and TFE). It should be noted that cytochrome *c* starts to aggregate when concentration of the organic solvent goes beyond 50% IPA, 60% *t*-BuOH, or 60% acetonitrile. The secondary structure of lysozyme appears to be more rigid than that of cytochrome *c*, yet is less stable than apoNCS. The far-UV CD signal of lysozyme at 222 nm remains unchanged up to concentration of 60% methanol, 60% ethanol, 50% IPA, 45% *t*-BuOH, 60% acetonitrile, or 20% TFE. Lysozyme starts to aggregate beyond concentration of 60% acetonitrile, 75% IPA, or 75% *t*-BuOH. When unfolding of lysozyme is studied against increasing concentration of acetonitrile, the aggregation occurs before any conformational changes are observed. Hence, no stability comparison can be made between apoNCS and lysozyme against acetonitrile. Results from cytochrome *c* and lysozyme are summarized in Table 1, where we listed the number of disulfide bonds in each protein. Lysozyme consists of four disulfide bonds and presumably is more stable than cytochrome *c*, which has no disulfide bond. Considering that apoNCS contains only two disulfide bonds, the extraordinary high stability of apoNCS probably results from its own inherent structural peculiarities rather than disulfide linkages.

2.1.2. Effect of chemical denaturants. Urea and GdnHCl are well-known denaturants and are conveniently used in this study for assessing the conformational stability of apoNCS. Figure 3 shows the urea- and GdnHCl-induced unfolding curves, monitored by far-UV CD at 25 °C and pH 5. The CD signal at 224 nm shows no changes below 6 M urea or 3.5 M GdnHCl. Beyond these concentrations, the native structure of apoNCS starts to melt, as shown by the decreasing of the CD signal at 224 nm. However, apoNCS does not fully denature even in the presence of 9 M urea or 6 M GdnHCl, as indicated by the incomplete denaturation curves shown in Figure 3. The C_m values are estimated to be larger than 7.3 and 4.9 M for urea and GdnHCl, respectively. Earlier reports[15,16] showed that, at either 12 or 4 °C, apoNCS retains its native-like conformation only up to 2 M GdnHCl. At these temperatures the protein fully denatures at a concentration below 4 M, giving C_m values of 2.8–3.0 M. We reported recently that apoNCS has a tendency of cold denaturation.[14] The instability of apoNCS at low temperatures is expected to lower C_m of denaturants substantially. On the other hand, our results of the urea- and GdnHCl-induced unfolding of apoNCS are compatible with an earlier observation, in which both urea and GdnHCl were found not to be effective in assisting disulfide reduction of NCS.[6] The urea-induced unfolding profile of apoNCS monitored by far-UV CD at pH 5 (Fig. 3) is

Table 1. Comparison of conformational stability of apoNCS with other stable proteins of similar size

Proteins	Length (no. of aa)	Disulfide bonds	Secondary structure	C_m (%(v/v) or M)	Conditions	References
1. Methanol-induced conformational changes						
ApoNCS	113	2	β	⩾78%[a]	25 °C, pH 5	This study
Cytochrome *c*	104	0	α	56%[b]	25 °C, pH 5	This study
Lysozyme	129	4	α/β	69%[c]	25 °C, pH 5	This study
Cytochrome *c*	104	0	α	51%	25 °C, pH 5.7	28
Myoglobin	153	0	α	50%	25 °C, pH 5.7	28
β-Lactoglobulin	162	2	β	47%[d]	20 °C, pH 2 (stable at pH 2)	29
2. Ethanol-induced conformational changes						
ApoNCS	113	2	β	>76%[a]	25 °C, pH 5	This study
Cytochrome *c*	104	0	α	44%[b]	25 °C, pH 5	This study
Lysozyme	129	4	α/β	68%[c]	25 °C, pH 5	This study
Cytochrome *c*	104	0	α	42%	25 °C, pH 5.7	28
Myoglobin	153	0	α	31%	25 °C, pH 5.7	28
β-Lactoglobulin	162	2	β	32%[d]	20 °C, pH 2 (stable at pH 2)	29
α-Lactalbumin	123	4	α/β	45%[e]	20 °C, pH 8	30
3. Isopropanol (IPA)-induced conformational changes						
ApoNCS	113	2	β	>76%[a]	25 °C, pH 5	This study
Cytochrome *c*	104	0	α	28%[b]	25 °C, pH 5	This study
Lysozyme	129	4	α/β	63%[c]	25 °C, pH 5	This study
Cytochrome *c*	104	0	α	35%	25 °C, pH 5.7	28
Myoglobin	153	0	α	26%	25 °C, pH 5.7	28
β-Lactoglobulin	162[f]	2	β	27%[d]	20 °C, pH 2 (stable at pH 2)	29
Glycodelin A	162[f]	2	β	27%	25 °C w/o buffer	31
β-Lactoglobulin A	162	2	β	31%	25 °C w/o buffer	31
4. Acetonitrile-induced conformational changes						
ApoNCS	113	2	β	>80%[a]	25 °C, pH 5	This study
Cytochrome *c*	104	0	α	30%[b]	25 °C, pH 5	This study
Lysozyme	129	4	α/β	>60%[c]	25 °C, pH 5	This study
Lysozyme	129	4	α/β	>50%[g]	25 °C, pH 3	32
5. tert-Butyl alcohol (t-BuOH)-induced conformational changes						
ApoNCS	113	2	β	>75%[a]	25 °C, pH 5	This study
Cytochrome *c*	104	0	α	36%[b]	25 °C, pH 5	This study
Lysozyme	129	4	α/β	59%[c]	25 °C, pH 5	This study
Cytochrome *c*	104	0	α	34%	25 °C, pH 5.7	28
Myoglobin	153	0	α	23%	25 °C, pH 5.7	28
β-Lactoglobulin	162	2	β	18%[d]	20 °C, pH 2 (stable at pH 2)	29
6. TFE-induced conformational changes						
ApoNCS	113	2	β	45%	25 °C, pH 5	19
Cytochrome *c*	104	0	α	30%[b]	25 °C, pH 5	This study
Lysozyme	129	4	α/β	30%[c]	25 °C, pH 5	This study
β-Lactoglobulin	162	2	β	17%[d]	20 °C, pH 2 (stable at pH 2)	29
Lysozyme	129	4	α/β	36%	25 °C, pH 5.2	33
Stefin A	98	0	α/β	39%	20 °C, pH 6.5[h]	34
Stefin B (cystatin B)	98	0	α/β	26%	20 °C, pH 6.5[h]	34
Ribonuclease A	124	4	α/β	48%	25 °C w/o buffer	35
α-Lactalbumin	123	4	α/β	18%	25 °C w/o buffer	35
7. Urea-induced conformational changes						
ApoNCS	113	2	β	>7.3 M[a]	25 °C, pH 5	This study
Cytochrome *c*	104	0	α	6.6 M	25 °C, pH 5.7	36
Myoglobin	153	0	α	6.6 M	25 °C, pH 5.7	36
Lysozyme	129	4	α/β	5.21 M	25 °C, pH 2.9	37
Ribonuclease A	124	4	α/β	6.96 M	25 °C, pH 6.6	37
β-lactoglobulin	162	2	β	5.01 M	25 °C, pH 3.2	37
β-Lactoglobulin	162	2	β	5.1 M	24.8 °C, pH 2.77	38
Ribonuclease T_1	104	2	α/β	4.33 M	25 °C, pH 7	39
Barnase	110	0	α/β	4.49 M	25 °C, pH 7	40
Myoglobin (horse)	153	0	α	5.40 M	25 °C, pH 7.5	41
Myoglobin (sperm whale)	154	0	α	7.20 M	25 °C, pH 7.5	41
8. GdnHCl-induced conformational changes						
ApoNCS	113	2	β	>4.9 M[a]	25 °C, pH 5	This study
Lysozyme	129	4	α/β	3.07 M	25 °C, pH 2.9	37

Table 1 (*continued*)

Proteins	Length (no. of aa)	Disulfide bonds	Secondary structure	C_m (%(v/v) or M)	Conditions	References
Ribonuclease A	124	4	α/β	3.01 M	25 °C, pH 6.6	[37]
β-Lactoglobulin	162	2	β	3.23 M	25 °C, pH 3.2	[37]
Myoglobin (horse)	153	0	α	1.63 M	25 °C, pH 7–8	[41]
Myoglobin (sperm whale)	154	0	α	2.36 M	25 °C, pH 7–8	[41]
Ribonuclease A	124	4	α/β	2.98 M	25 °C, pH 6.98	[42]
Ribonuclease T_1	104	2	α/β	2.99 M	25 °C, pH 6.98	[42]
Lysozyme	129	4	α/β	2.94 M	25 °C, pH 3	[43]
Cytochrome *c* (cow)	107	0	α	2.63 M	25 °C, pH 6.5	[44]
Cytochrome *c* (horse)	104	0	α	2.42 M	25 °C, pH 6.5	[44]
Cytochrome *c* (candida)	109	1	α	1.89 M	25 °C, pH 6.5	[44]
α-Lactalbumin	123	4	α/β	2.75 M	25 °C, pH 6.65	[45]
9. SDS-induced conformational changes						
ApoNCS	113	2	β	>887 mM[i]	25 °C, pH 7	This study
Cytochrome *c*	104	0	α	0.6 mM	25 °C[j], pH 7.4	[46]
Lysozyme (human bronchial)	129	4	α/β	25 mM[k]	20 °C, pH 7	[47]
Myoglobin	153	0	α	2 mM[l]	25 °C, pH 7	[48]

[a] The conformational changes were monitored by CD spectroscopy at both far-UV at 224 nm and near-UV at 270 nm using 10 μM apoNCS in a mixed buffer of 20 mM of sodium acetate and 2.67 mM of ammonium acetate at pH 5. Most cases the transitions of apoNCS are not complete at the highest concentration of the denaturant. The C_m values are estimated from the midpoint of the available transition data and may be lower than the actual values.

[b] The conformational changes were monitored by CD spectroscopy at 208 nm using 10 μM cytochrome *c* in a mixed buffer of 20 mM sodium acetate and 2.67 mM ammonium acetate at pH 5.

[c] The conformational changes were monitored by CD spectroscopy at 222 nm using 10 μM lysozyme in a mixed buffer of 20 mM sodium acetate and 2.67 mM ammonium acetate at pH 5.

[d] For consistency, the m values shown in the referred report[29] are converted to C_m in v/v percentage using an equation of $\Delta G_D = mC_m$ and an experimental value of 23.3 kJ/mol for ΔG_D.

[e] For consistency, the X_A values shown in the referred report[30] are converted to C_m in v/v percentage.

[f] The protein is a dimer at pH 7.0.[49]

[g] The CD spectra of lysozyme were reported only up to 60% acetonitrile, in which the protein appears to be not at a denatured state (see Fig. 4 in the referred report).[32] The 50% concentration of acetonitrile is the midpoint of the incomplete transitions reported.

[h] The temperature was not clearly stated in the referred report.[34] Since the same reference indicated that the kinetics experiments were performed at 20 °C, pH 6, we assume that the conformational transitions were also recorded at 20 °C.

[i] The conformational changes were monitored by CD spectroscopy using 10 μM apoNCS in 20 mM of sodium phosphate, pH 7. The CD signals of apoNCS show no significant changes up to 887 mM of SDS, which is the maximum concentration allowed by CD measurement.

[j] The temperature was not clearly stated in the unfolding experiments in the referred report.[46] Since the same reference indicated that the unfolding kinetics was performed at 298 K, we assume that the conformational transitions were also recorded at 25 °C.

[k] It is not clear what C_m value should be in the referred report.[47] The highest concentration of SDS studied by the authors was 25 mM, in which the CD signals of lysozyme at 222 nm decrease sharply to twofold (from a value of -4.1×10^3 deg cm^2 dmol^{-1} obtained in 1.25 mM SDS to a value of -7.7×10^3 deg cm^2 dmol^{-1} in 25 mM SDS) (estimated from Fig. 5 in this referred report[47]).

[l] The C_m value is estimated based on the surface tension curve shown in Figure 2 in the referred report.[48]

superimposable with that observed at pH 7.[14] The transition profile monitored at 224 nm, though incomplete, overlaps with the one monitored by near-UV CD at 270 nm.[14] The results imply that both secondary and tertiary structures of apoNCS exhibit equal resistance to unfolding against urea.

Detergents such as SDS, by virtue of their amphipathic property mimics molecules in cell membrane, are very effective protein denaturants. SDS is in general 500–1000 times more effective than urea and GdnHCl in protein denaturation.[20] An earlier report[21] showed that SDS at 100 mM concentration causes no changes in the CD profile of apoNCS. Strikingly, we did not observe any significant changes in the CD spectrum of apoNCS in the presence of SDS at a concentration 8-fold higher than the former. Figure 4 depicts the appreciable superimposability of both far- and near-UV CD spectra of apoNCS in 887 mM SDS on those of its native form at 25 °C and pH 7. The high viscosity of SDS solutions does not allow proper observation by CD spectroscopy when the concentration is beyond 887 mM (~25.3% w/v).

2.2. Conformational stability analyzed by NMR spectroscopy

1D ^{1}H NMR is the simplest, but sensitive, NMR method to distinguish the molecular structures of folded and unfolded forms of a protein. The apoNCS in aqueous solution shows narrow peaks with good dispersion in its 1D ^{1}H NMR spectrum (Fig. 5A), reflecting its compactly folded native structure. Titration of organic solvents does not significantly perturb chemical shifts and dispersion quality until very high concentrations. Figure 5A shows two examples of 1D ^{1}H NMR analyses on probing the stability of apoNCS at 25 °C and pH 7 against organic solvents (one example, in 72% acetonitrile, and the other, 50% methanol). The ^{1}H resonance signals of apoNCS are found to remain fairly dispersed

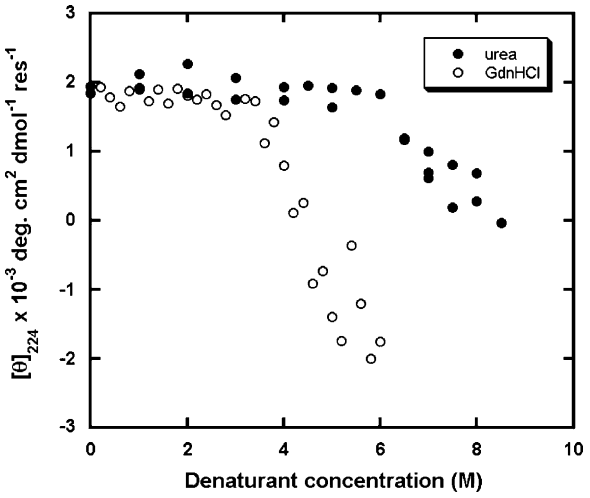

Figure 3. The melting profile of apoNCS monitored at 25 °C and pH 5 by changes in the mean residual ellipticity [θ] of far-UV CD at 224 nm with respect to increasing molar concentration of GdnHCl (○) and urea (●).

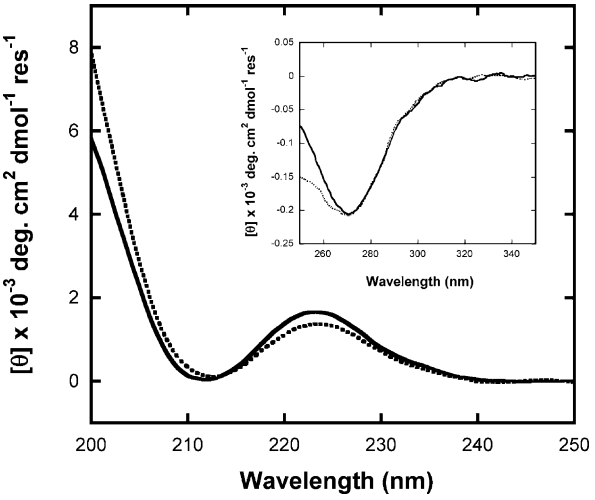

Figure 4. Comparison of the CD spectrum of apoNCS observed at 25 °C in 20 mM sodium phosphate, pH 7, between in aqueous solution (—) and in the presence of 887 mM SDS (···). The spectrum is expressed by mean residue ellipticity [θ] of apoNCS. The inset shows the near-UV CD spectra of apoNCS in these two conditions.

in both spectra. These include aromatic (6–8 ppm) and amide (7–10 ppm) regions. The results suggest that the specific inter-residue interactions among aromatic side chains of apoNCS remain unchanged and the protein retains its native-like compact structure in these organic solvents, a condition that is in general unfavorable to most folded proteins.

Close inspection on the aliphatic region of 1D ^{1}H NMR spectrum of apoNCS reveals that chemical shifts of some methyl peaks are at up field below 0 ppm. The negative resonances indicate that these highly shielded aliphatic side chains are well packed in a very specific manner in the hydrophobic core of apoNCS. Interestingly, these methyl peaks retain their negative values of chemical shift in organic solvents (Fig. 5A), indicating that the specific hydrophobic packing in apoNCS is undisturbed by organic denaturants.

While 1D NMR spectroscopy provides qualitative information on the stability of the folded protein structure under the NMR conditions, 2D NMR spectroscopy reveals the extent of structural changes in polypeptide backbone. In particular, ^{15}N–^{1}H HSQC provides a fingerprint for a protein at different structural states. To closely examine the conformational stability against denaturants at the residue level, we prepared the ^{15}N-labeled recombinant apoNCS from ^{15}N enriched culture. The purity of ^{15}N apoNCS (>95%) is examined by SDS–PAGE, UV, and HPLC, and the identity of the protein is confirmed by CD and mass spectrometry.

The ^{1}H–^{15}N HSQC spectrum of the native form of ^{15}N apoNCS in aqueous buffer at pH 7 and 25 °C is shown in Figure 5B. The chemical shifts are in good agreement with those reported.[10] All cross peaks are well dispersed, indicating the compactness of the folded structure. Titration of organic solvents does not alter the dispersive quality, suggesting that the compact folded architecture of apoNCS is not affected. Close comparison of the ^{15}N–^{1}H HSQC resonance map of apoNCS obtained in aqueous solution (Fig. 5B, Native) with that obtained in 28% TFE, 50% methanol, or 40% acetonitrile (Fig. 5C) shows that overall pattern is unchanged in these solvents. Detailed chemical shift analyses for each individual residue reveal that changes of resonance are mostly below 0.2 ppm, which is considerably small. For instance, among 90 residues of apoNCS identified in the spectrum obtained in 28% TFE, only 14S, 40V, 54S, 77L, and 98S show perturbations larger than 0.5 ppm and none of residues larger than 1 ppm. Few examples of the residues whose resonance remains unchanged from the native state are labeled in Figure 5C.

3. Discussion

Proteins are known to be marginally stable at room temperature and all types of molecular interactions are important to stability.[22] The net difference between the free energies of the folded and unfolded forms is small, that is, 5–20 kcal/mol. The understanding of protein stability is therefore complicated by the necessity to account for trivial free energy changes associated with folding–unfolding transitions.[23] In this study, we evaluate the stability of apoNCS as a function of conformational properties of the native folded and the denaturant-induced states.

3.1. Evaluation of the structural endurance of apoNCS by C_{min} values

To assess the structural endurance of apoNCS, conformational transitions are analyzed by titration with denaturants. For comparison, lysozyme, and cytochrome *c*, known to be stable and similar in size to apoNCS, are also examined side by side with apoNCS. Most cases, the fully denatured state of apoNCS cannot be induced by these denaturants at the nearly maximum concentra-

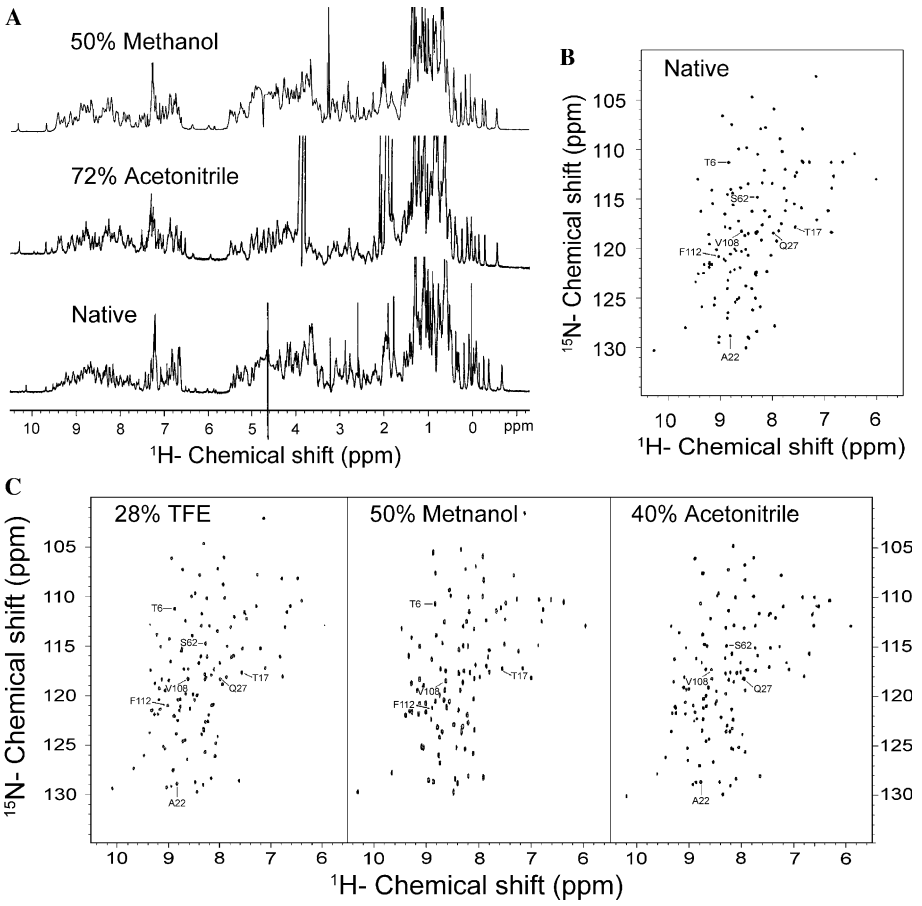

Figure 5. (A) 1D ^{1}H NMR spectra of apoNCS recorded at 25 °C in 20 mM sodium phosphate, pH 7. The spectra shown are in aqueous solution of 90% H_2O/D_2O, 72% acetonitrile-d_3/H_2O (v/v), and 50% methanol-d_3/H_2O (v/v); (B) 2D ^{1}H–^{15}N HSQC NMR spectrum of native apoNCS recorded at 25 °C in 20 mM sodium phosphate, pH 7, in an aqueous solution of 90% H_2O/D_2O; (C) 2D ^{1}H–^{15}N HSQC NMR spectra of apoNCS recorded at 25 °C in 20 mM sodium phosphate, pH 7, in 28% TFE-d_3/H_2O (v/v), 50% methanol-d_4/H_2O (v/v), and 40% acetonitrile-d_3/H_2O (v/v). The labeled residues are few examples that show no changes in chemical shift by comparing with the spectrum of native apoNCS in aqueous solution (shown in (B)).

tion. Thus, the C_m values of apoNCS are difficult to measure accurately and cannot be used for the stability comparison with two other proteins. We therefore evaluate structural stability of the proteins using C_{min} values, which is designated to represent the lowest denaturant concentration required to induce any structural changes of the protein and can be estimated easily from the incomplete transition profiles. By comparing C_{min} values among three proteins studied under the same condition, it is apparent that apoNCS exhibits the highest structural stability. If we consider the capability of apoNCS to retain its structural integrity against each type of denaturant is 1.00, the structural endurance of lysozyme, as estimated by ratios of C_{min} values, is only 0.86, 0.92, 0.83, 0.75, and 0.80 against methanol, ethanol, IPA, *t*-BuOH, and TFE, respectively. Similarly, the structural endurance of cytochrome *c* is only 0.64, 0.38, 0.25, 0.25, 0.16, and 0.60, against methanol, ethanol, IPA, *t*-BuOH, acetonitrile, and TFE, respectively. These values are substantially smaller than that of apoNCS. The results suggest that apoNCS exhibits unusually high stability against organic solvents. The conclusion is consistent with the observation from 1D ^{1}H and 2D ^{15}N–^{1}H HSQC NMR studies.

3.2. Comparison of the apoNCS stability with stable proteins of similar size

Table 1 summarizes the comparison of the stability data for apoNCS with several known stable proteins (similar in size to apoNCS) available in literatures. For comparison purpose, we unified the units of C_m used in each report. The data presented in Table 1 are organized based on the type of the denaturant. Some of the stability-related biophysical details are included to impart good comprehension. Table 1 shows that our stability data for cytochrome *c*, as observed in methanol-, ethanol-, IPA-, and *t*-BuOH-induced changes, are relatively consistent with those reported elsewhere, suggesting the validity of our data. Table 1 clearly demonstrates that apoNCS exhibits far more rigidity than most other stable proteins such as myoglobin or β-lactoglobulin in organic solvents such as methanol, ethanol, IPA, and *t*-BuOH. The C_m of apoNCS in TFE is slightly lower than that of ribonuclease A, but is still far higher than those of other proteins.

Chaotropic agents such as urea and GdnHCl denature proteins in general at concentrations of 6–8 and 4–6 M, respectively.[20] The C_m of sperm whale myoglobin

in urea-induced transitions is very high (7.2 M) (see Table 1), yet it is smaller than that of apoNCS, whose fully denatured state cannot be induced at the maximum concentration of urea. Similarly, apoNCS remains partially structured in a solution of 6 M GdnHCl (C_m > 4.9 M), while most known stable proteins denature at much lower concentrations (C_m < 4 M).

The anionic detergent SDS is a powerful protein denaturant with its hydrophobic tail that can penetrate the interior of proteins and disrupt the hydrophobic interaction therein. Except a few cases such as papain and pepsin, the anionic detergent SDS can effectively unfold most proteins at a concentration below 8 mM[20] (see Table 1 for a few examples). The structural resistance of apoNCS is extraordinary high against SDS, which induces no changes at a concentration of 887 mM. The C_m value is strikingly higher than those of cytochrome *c*, myoglobin, and lysozyme.

3.3. Biological significance of the high stability of apoNCS

Compared to most proteins, the unusually high resistance of apoNCS against conformational changes induced by denaturants at ambient temperatures is quite unique. This unusually high stability of apoNCS against denaturants must bear some biological significance. The apoNCS is known to be a protector and carrier of the labile toxin, NCS-C. Considering that the labile NCS-C needs to be well preserved during the drug delivery time course, it is probably natural for apoNCS, which is designed as a matched safeguard, to be rigid in conformational changes against chemicals. Our results demonstrate that apoNCS fulfills one of the fundamental criteria of being a superior drug carrier.

Biological system is highly complex and the defense components of the biological systems are highly multifarious. Though apoNCS is stable against many chemical denaturants, it is conceivable that it may encounter a number of other challenges like proteases posed by the host cells. Interestingly, early studies on NCS[6,24] showed that native NCS resists digestion by proteases like trypsin or chymotrypsin. Our preliminary results[†] also show that apoNCS is highly resistant to the digestion by V8 protease. ApoNCS remains intact after 15–24 h incubation with V8 protease in the presence of 1–1.5 M GdnHCl at pH 7.8 and 37 °C. These results indicate that the stability of apoNCS against proteases, which is beyond the scope of this study, is unusually high as well.

It should be noted that the interactions between a carrier protein and a drug may involve specific orientation of side chains as well. In addition to major backbone changes, minor conformational changes can also interfere with the drug protecting function of apoNCS. Our conclusion that apoNCS is highly resistant to chemical-induced changes, drawn from major backbone structural changes, cannot be freely extended to minor changes. The possibility of minor or side-chain conformational changes in apoNCS induced by chemicals cannot be ruled out. Some experimental evidences indicate such fluctuations or minor conformational changes in apoNCS may interfere in its binding to NCS-C. For instance, 20 mM SDS was reported[25] to cause release of NCS-C from holoNCS, while backbone conformation of apoNCS remains unchanged in a solution of 887 mM SDS. We also demonstrated earlier[19] that the release of NCS-C from holoNCS is sharply stimulated by 15% TFE, while the compact folded tertiary structure of apoNCS remains intact up to concentration of 20% TFE.

[†] Unpublished data by Chin-Jui Tseng, Parameswaran Hariharan, and Der-Hang Chin.

4. Experimental

4.1. Materials

Methanol, ethanol, IPA, acetonitrile, TFE, and *t*-BuOH were obtained from Sigma-Aldrich, St. Louis, MO, USA. The ingredients for the LB medium, bacto-tryptone, and yeast extract were obtained from Difco laboratories, USA. Deuterated NMR solvents were obtained from Cambridge Isotope Laboratories, Inc., Andover, MA, USA. Horse heart cytochrome *c*, hen egg lysozyme, V8 protease, ampicillin, β-mercaptoethanol, and urea were also obtained from Sigma-Aldrich. SDS (ultrapure) was purchased from MP Biomedicals, Irvine, CA, USA. GdnHCl (biotechnology grade) was purchased from Amresco Inc., Solon, Ohio, USA. All protein solutions are prepared using 18.2 ohm Milli-Q water and the pH is adjusted by buffers before making any spectroscopic measurements.

4.2. Expression and purification of the recombinant apoNCS

The expression construct of apoNCS in pCAL-n-EK vector[26] is transformed into *Escherichia coli* BL21 Codon Plus (Stratagene, La Jolla, CA, USA) expression strain. Isopropyl-thio-β-D-galactopyranoside (1 mM) is used to induce the expression of the apoNCS-calmodulin binding peptide (CBP) fusion protein. The apoNCS-CBP fusion protein is then purified by binding into the calmodulin affinity resin (Stratagene, La Jolla, CA, USA) in the presence of 2 mM $CaCl_2$ and then eluting with 2 mM EDTA. Recombinant enterokinase (Ekmax; Invitrogen, CA, USA) or porcine enterokinase (GenScript, Piscataway, NJ, USA) is used to remove the CBP tag, to obtain apoNCS without any extra amino acid in termini. Finally DEAE-Sepharose (Fast Flow) resin (Amersham Pharmacia Biotech AB, Uppsala, Sweden) is used to get pure and homogeneous apoNCS protein. The purity, which is greater than 95% in an average, is examined by UV, CD, electrophoresis, high-performance liquid chromatography (HPLC) analyses, and mass spectroscopy (for detail, see[26]).

4.3. Preparation of ^{15}N isotopically enriched apoNCS

^{15}N isotope labeling of apoNCS is achieved by growing *E. coli* BL21 Codon Plus strain carrying pCAL-n-EK

vector[26] in M9 minimal medium containing 0.125 or 0.250 g/L of $^{15}NH_4Cl$ (Cambridge Isotope Laboratories, Inc., MA, USA). Vitamin B1 is supplemented to the medium, since the expression strain is a vitamin B1-deficient host. Subsequently, ^{15}N-labeled apoNCS is purified following the procedure used for purifying unlabeled apoNCS (for detail, see[26]).

4.4. CD spectroscopy

All CD measurements are carried out on a Jasco J-715 spectropolarimeter (Tokyo, Japan) equipped with a circulating water bath (Neslab, model RTE-140) (Portsmouth, NH, USA). The instrument is calibrated with ammonium *d*-10-camphor sulfonate. A 1 mm water-jacketed cell is used for far-UV (190–250 nm) CD measurements and 10 mm quartz micro cell for near-UV (240–320 nm). Protein samples were prepared either in a mixed buffer of 2.67 mM ammonium acetate and 20 mM sodium acetate at pH 5 or in 20 mM phosphate buffer at pH 7. The denaturant-induced transitions were monitored at 25 °C with a scan speed of 50 nm/min using samples of 10 μM apoNCS in requisite concentrations of organic solvents (expressed as percentage volume to volume, v/v) or chemical reagents (expressed as molar concentration). All spectra are corrected for solvent and buffer blanks.

4.5. NMR spectroscopy

1D ^1H NMR spectra were recorded with a spectral sweep width of 11,000 Hz at 25 °C on either a Varian 600 MHz NMR (Palo Alto, CA, USA) or a Bruker DMX 600 MHz spectrometer (Rheinstetten, Germany). 2D ^{15}N–^1H HSQC NMR experiments were carried out on a Bruker DMX 600 MHz spectrometer at 25 °C. An inverse probe with a self-shielded *z*-gradient was used to obtain all gradient-enhanced ^1H–^{15}N HSQC spectra.[27] A total of 2048 complex data points were collected in the ^1H dimension, and 512 complex data points were collected in the indirect ^{15}N dimension. The ^{15}N-labeled apoNCS, 6 mg/mL (0.54 mM), in 20 mM sodium phosphate, pH 7, was lyophilized and then dissolved in 95% H_2O/5% D_2O containing requisite amount of TFE-d_3, methanol-d_4 or acetonitrile-d_3. The 2D spectra were recorded by 36 scans for each denaturant-subjected condition and all data were processed using XWINNMR and Sparky software. The cross peaks for native apoNCS are identified based on the reported assignments[10] using Try32 as an internal reference. The assignment in TFE was done by tracing small changes from titration of TFE with small increment (3–5% TFE each step). For 1D NMR experiments, appropriate amount of apoNCS in 20 mM sodium phosphate, pH 7, was lyophilized and then dissolved in a mixture of 90% H_2O and 10% D_2O containing requisite amount of TFE-d_3, acetonitrile-d_3 or methanol-d_4.

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References and notes

1. Ishida, N.; Miyazaki, K.; Kumagai, K.; Rikimaru, M. *J. Antibiot.* **1965**, *18*, 68.
2. Edo, K.; Mizugaki, M.; Koide, Y.; Seto, H.; Furihata, K.; Otake, N.; Ishida, N. *Tetrahedron Lett.* **1985**, *26*, 331.
3. Goldberg, I. H. *Acc. Chem. Res.* **1991**, *24*, 191.
4. Xi, Z.; Goldberg, I. H.. In *Comprehensive Natural Products Chemistry*; Barton, D. S., Nakanishi, K., Eds.; Elsevier: New York, 1999; Vol. 7, pp 553–592.
5. Goldberg, I. H.; Kappen, L. S. In *Enediyne Antibiotics as Antitumor Agents*; Borders, D. B., Doyle, T. W., Eds.; Marcel Dekker: Hong Kong, 1995; pp 326–327.
6. Meienhofer, J.; Maeda, H.; Glaser, C. B.; Czombos, J.; Kuromizu, K. *Science* **1972**, *178*, 875.
7. Teplyakov, A.; Obmolova, G.; Wilson, K.; Kuromizu, K. *Eur. J. Biochem.* **1993**, *213*, 737.
8. Kim, K. H.; Kwon, B. M.; Myers, A. G.; Rees, D. C. *Science* **1993**, *262*, 1042.
9. Chin, D.-H. *Chem. Eur. J.* **1999**, *5*, 1084.
10. Urbaniak, M. D.; Muskett, F. W.; Finucane, M. D.; Caddick, S.; Woolfson, D. N. *Biochemistry* **2002**, *41*, 11731.
11. Mohanty, S.; Sieker, L. C.; Drobny, G. P. *Biochemistry* **1994**, *33*, 10579.
12. Urbaniak, M. D.; Bingham, J. P.; Hartley, J. A.; Woolfson, D. N.; Caddick, S. *J. Med. Chem.* **2004**, *47*, 4710.
13. Heyd, B.; Pecorari, F.; Collinet, B.; Adjadj, E.; Desmadril, M.; Minard, P. *Biochemistry* **2003**, *42*, 5674.
14. Jayachithra, K.; Kumar, T. K. S.; Lu, T. J.; Yu, C.; Chin, D.-H. *Biophys. J.* **2005**, *88*, 4252.
15. Russo, D.; Durand, D.; Calmettes, P.; Desmadril, M. *Biochemistry* **2001**, *40*, 3958.
16. Nicaise, M.; Valerio-Lepiniec, M.; Izadi-Pruneyre, N.; Adjadj, E.; Minard, P.; Desmadril, M. *Protein Eng.* **2003**, *16*, 733.
17. Kappen, L. S.; Goldberg, I. H. *Nucleic Acids Res.* **1978**, *5*, 2959.
18. Kappen, L. S.; Goldberg, I. H. *Biochemistry* **1979**, *18*, 5647.
19. Sudhahar, G. C.; Balamurugan, K.; Chin, D.-H. *J. Biol. Chem.* **2000**, *275*, 39900.
20. Jones, M. N.; Chapman, D. In *Micelles Monolayers and Biomembranes*; Jones, M. N., Chapman, D., Eds.; Wiley-Liss: New York, 1995; pp 150–151.
21. Saito, K.; Sato, Y.; Edo, K.; Akiyama-Murai, Y.; Koide, Y.; Ishida, N.; Mizugaki, M. *Chem. Pharm. Bull.* **1989**, *37*, 3078.
22. Dill, K. A. *Biochemistry* **1990**, *29*, 7133.
23. Matthews, B. W. *Biochemistry* **1987**, *26*, 6885.
24. Maeda, H.; Kumagai, K.; Ishida, N. *J. Antibiot.* **1966**, *19*, 253.
25. Edo, K.; Saito, K.; Akiyama-Murai, Y.; Mizugaki, M.; Koide, Y.; Ishida, N. *J. Antibiot.* **1988**, *41*, 554.
26. Hariharan, P.; Liang, W.; Chou, S.-H.; Chin, D.-H. *Nat. Chem. Biol.*, submitted for publication.

27. Palmer, A. G.; Cavanagh, J.; Wright, P. E.; Rance, M. *J. Magn. Reson.* **1991**, *93*, 151.
28. Herskovits, T. T.; Gadegbeku, B.; Jaillet, H. *J. Biol. Chem.* **1970**, *245*, 2588.
29. Hirota-Nakaoka, N.; Goto, Y. *Bioorg. Med. Chem.* **1999**, *7*, 67.
30. Grinberg, V. Y.; Grinberg, N. V.; Burova, T. V.; Dalgalarrondo, M.; Haertle, T. *Biopolymers* **1998**, *46*, 253.
31. Gaudiano, M. C.; Pala, A.; Barteri, M. *Biochim. Biophys. Acta* **1999**, *1431*, 451.
32. Gekko, K.; Ohmae, E.; Kameyama, K.; Takagi, T. *Biochim. Biophys. Acta* **1998**, *1387*, 195.
33. Hoshino, M.; Hagihara, Y.; Hamada, D.; Kataoka, M.; Goto, Y. *FEBS Lett.* **1997**, *416*, 72.
34. Zerovnik, E.; Virden, R.; Jerala, R.; Kroon-Zitko, L.; Turk, V.; Waltho, J. P. *Proteins* **1999**, *36*, 205.
35. Sivaraman, T.; Kumar, T. K. S.; Yu, C. *Int. J. Biol. Macromol.* **1996**, *19*, 235.
36. Herskovits, T. T.; Jaillet, H.; Gadegbeku, B. *J. Biol. Chem.* **1970**, *245*, 4544.
37. Greene, R. F.; Pace, C. N. *J. Biol. Chem.* **1974**, *249*, 5388.
38. Pace, N. C.; Tanford, C. *Biochemistry* **1968**, *7*, 198.
39. Pace, C. N.; Grimsley, G. R.; Thomson, J. A.; Barnett, B. J. *J. Biol. Chem.* **1988**, *263*, 11820.
40. Pace, C. N.; Laurents, D. V.; Erickson, R. E. *Biochemistry* **1992**, *31*, 2728.
41. Puett, D. *J. Biol. Chem.* **1973**, *248*, 4623.
42. Pace, C. N.; Laurents, D. V.; Thomson, J. A. *Biochemistry* **1990**, *29*, 2564.
43. Pace, C. N.; Mcgrath, T. *J. Biol. Chem.* **1980**, *255*, 3862.
44. Knapp, J. A.; Pace, C. N. *Biochemistry* **1974**, *13*, 1289.
45. Kuwajima, K.; Nitta, K.; Yoneyama, M.; Sugai, S. *J. Mol. Biol.* **1976**, *106*, 359.
46. Das, T. K.; Mazumdar, S.; Mitra, S. *Eur. J. Biochem.* **1998**, *254*, 662.
47. Van-Seuningen, I.; Hayem, A.; Davril, M. *Int. J. Biochem.* **1992**, *24*, 593.
48. Tofani, L.; Feis, A.; Snoke, R. E.; Berti, D.; Baglioni, P.; Smulevich, G. *Biophys. J.* **2004**, *87*, 1186.
49. Barteri, M.; Gaudiano, M. C.; Rotella, S.; Benagiano, G.; Pala, A. *Biochim. Biophys. Acta* **2000**, *1479*, 255.