DOI: 10.1002/chem.200800855

Organic Ligand Binding by a Hydrophobic Cavity in a Designed Tetrameric Coiled-Coil Protein

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Abstract: The design and characterization of a hydrophobic cavity in de novo designed proteins provides a wide range of information about the functions of de novo proteins. We designed a de novo tetrameric coiled-coil protein with a hydrophobic pocketlike cavity. Tetrameric coiled coils with hydrophobic cavities have previously been reported. By replacing one Leu residue at the *a* position with Ala, hydrophobic cavities that did not flatten out due to loose peptide chains were reliably created. To perform a detailed examination of the ligand-binding characteristics of the cavities, we originally designed two other coiled-coil proteins: AM2, with eight Ala substitutions at the adjacent *a* and *d* positions at the center of a bundled structure, and AM2W, with one Trp and seven Ala substitutions at the same positions. To increase the association of the helical peptides, each helical peptide was connected with flexible linkers, which resulted in a single peptide chain. These proteins exhibited CD spectra corresponding to superhelical structures, despite weakened hydrophobic packing. AM2W exhibited binding affinity for size-complementary organic compounds. The dissociation constants, K_d, of AM2W were 220 nM for adamantane, 81 μM for 1-adamantanol, and 294 μM for 1-adamantaneacetic acid, as measured by fluorescence titration analyses. Although it was contrary to expectations, AM2 did not exhibit any binding affinity, probably due to structural defects around the designed hydrophobic cavity. Interestingly, AM2W exhibited incremental structure stability through ligand binding. Plugging of structural defects with organic ligands would be expected to facilitate protein folding.

Keywords: ligand binding · protein design · protein structures

Introduction

The creation of a protein with a particular function is one of the goals of de novo protein design.[1–3] To design a functional protein, such as a receptor or an enzyme, sufficient disruption of hydrophobic packing is necessary to accommodate the substrate-binding site into the folded protein scaffold. The substrate-binding site usually requires a cavity or cleft (or concavity) that can be maintained without flattening out due to unfolding and solvation; partially defective structures would be expected to be less stable than fully folded structures.[4] Therefore, the number of examples, especially examples of the creation of pore structures in folded protein scaffolds, is quite small. To produce a de novo ligand-binding protein, the creation of a hydrophobic cavity in a de novo designed protein would be an effective approach and would provide a broad range of information about the de novo design of proteins. The selectivity of ligand binding, prescribed by size complementarity, is also an interesting characteristic that results in ligand-specific de novo proteins.[5]

Coiled-coil proteins are extensively studied de novo designed proteins with simple structures in which two–seven amphiphilic helices are associated together into a superhelical structure. While dimers to tetramers have mostly been

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studied in the past, only a few examples of coiled coils containing more than five monomers have been investigated.[6–8] Due to their good solubility in aqueous solution and the easier prediction of their tertiary structures from the peptide sequences, coiled-coil proteins have great potential in the examination of protein folding, interactions, and design templates for protein functions.[1a,2a,9] Recently, Yadav et al. reported that one Ala substitution in place of Leu at the *a* position of the GCN4-pLI peptide allowed the formation of tetrameric coiled-coil structures with a small cavity in the internal hydrophobic core.[10] Interestingly, from the crystallographic data, these cavities appear to avoid shrinkage after protein folding and to bind iodobenzene. However, there has, as yet, been no detailed examination of the ligand-binding characteristics, such as selectivity and thermodynamic parameters.

We designed other hydrophobic cavities, formed of more than four Ala substitutions at adjacent *a* and *d* positions, in de novo designed GCN4-pLI-based coiled-coil proteins, and we observed the characteristics of ligand binding with these cavities. By using isothermal titration calorimetry (ITC) and fluorescence titration, we examined the ligand selectivity, dissociation constants, and thermodynamic parameters. The size selectivity for the designed cavities was also tested by screening organic compounds. Gonzalez et al. reported the binding of benzene to a GCN4-based trimeric coiled-coil peptide with Ala substitutions in its hydrophobic core as an example of a ligand-binding coiled coil with a hydrophobic cavity.[11] They demonstrated that benzene plugged the cavity in the hydrophobic core and that the structure stability was thereby increased. We have also studied the effects of ligand binding on the ligand-binding cavity and the protein-structure stability.

Results

Tetrameric coiled coil with a hydrophobic cavity: α-Helical coiled coils have a representative, repeating, amino acid sequence $(abcdefg)_n$ heptad. The *a* and *d* positions are usually occupied by hydrophobic residues, and they form a hydrophobic core.[12] The GCN4-pLI peptide forms a parallel homotetrameric coiled-coil structure and maintains a folded tetrameric structure even above 95 °C.[7b] One substitution of Leu with Ala at the *a* position of GCN4-pLI peptide maintains the formation of a tetrameric coiled-coil structure with a small cavity in the internal hydrophobic core.[10] In this study, we increased the number of substitutions with Ala in the hydrophobic region. The individual peptides were connected with short flexible linkers, which resulted in a single polypeptide chain, to facilitate protein folding. The amino acid sequences of the designed coiled-coil proteins are summarized in Figure 1. The eight hydrophobic residues at the adjacent *a* and *d* positions at the center of the helical bundle were substituted with eight Ala residues or with one Trp and seven Ala residues; these proteins are denoted as AM2 and AM2W, respectively.

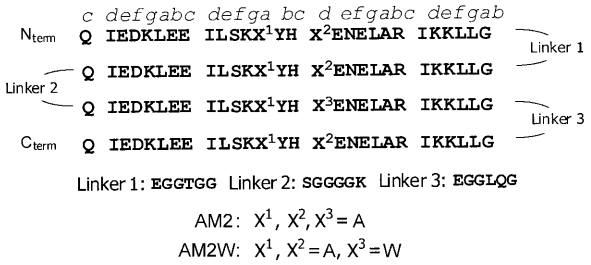


Figure 1. Amino acid sequences of AM2 and AM2W. X^1 and X^2 of the GCN4-pLI peptide (residues 3–29) are L and I, respectively. N_{term} and C_{term} are the N and C terminals, respectively.

In contrast to those in the work of Yadav et al., the four α-helical peptides of AM2 and AM2W required an antiparallel orientation, that is, an up–down–up–down structure. However, because the difference in the root-mean square deviation (rmsd) of the antiparallel- and parallel-oriented tetrameric coiled-coil structures of the GCN4-pLI sequences is reported to be only 0.68 Å, the stability energy gap between the parallel and antiparallel coiled coils might be small.[13] Therefore, a simple connection of four α-helical peptides with flexible linkers may be acceptable for the design of a single-chain tetrameric coiled-coil structure.

First, we examined the circular dichroism (CD) spectra to determine the secondary structures of the proteins.[14] The CD spectra of AM2 and AM2W were measured at pH 7.5 and 30 °C (Figure 2a). Spectral minima were observed at 208 and 222 nm, which are typical values for an α-helical structure. As the ratios of $[\theta]_{222}$ and $[\theta]_{208}$ were 1.0 for AM2 and 0.99 for AM2W, these proteins should take a coiled-coil structure. The temperature dependence of the $[\theta]_{222}$ value was examined by using a CD spectrometer equipped with a temperature-control apparatus over a range of 30 to 95 °C with intervals of 1 °C min^{-1}. AM2 exhibited thermal-denaturing behavior with a single transition and its melting temperature, T_m, was 85 °C, whereas AM2W began denaturing above 90 °C (Figure 2b). We therefore examined the thermal-denaturing behavior of AM2W in the presence of urea. As the urea concentration was increased, AM2W exhibited thermal-denaturing behavior below 95 °C (Figure 2c). Following increases in the urea concentration, increases of the $[\theta]$ value below the T_m value were also observed. This was likely due to the flexible linkers at the edge of the tetrameric bundle structures.[13] In the presence of 4 M urea, AM2W exhibited a T_m value of 75 °C, probably due to the destruction of the core structure of the designed tetrameric coiled coil containing a hydrophobic pore. Under the same conditions, AM2 almost lost its folded structure below 55 °C (Figure 2d).

Analytical ultracentrifugation is useful for the determination of macromolecular interactions, conformation changes, and molecular weights.[15,16] Since the GCN4-pIL peptide forms a tetrameric structure without the aid of links between each peptide, AM2 and AM2W might form intermolecular aggregates. We therefore examined the molecular weights of AM2 and AM2W by sedimentation equilibrium

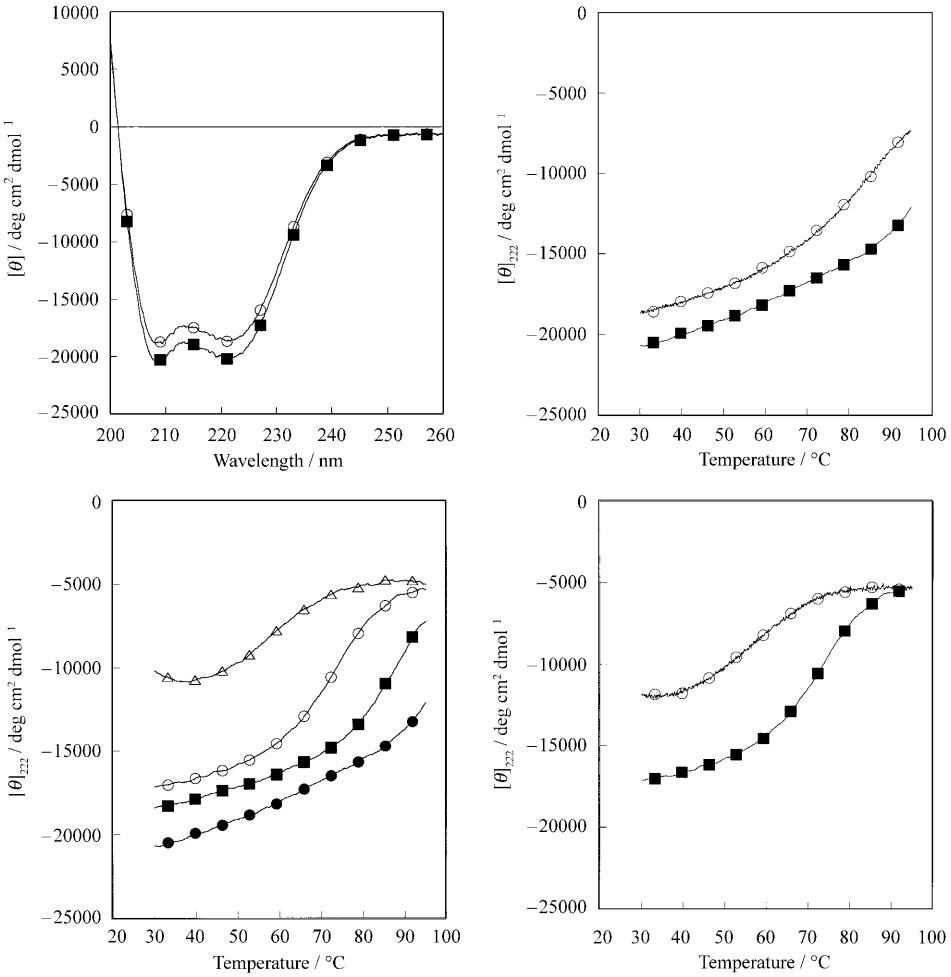


Figure 2. a) The CD spectra of AM2 (○), and AM2W (■): [protein] = 5 μM in 20 mM Tris-HCl buffer (pH 7.5) containing 100 mM NaCl. b) Temperature dependence of the $[\theta]_{222}$ value of AM2 (○) and AM2W (■): [protein] = 5 μM in 20 mM Tris-HCl buffer (pH 7.5) containing 100 mM NaCl. c) Temperature dependence of the $[\theta]_{222}$ value of AM2W in the absence and presence of urea (0–7.5 M): [AM2W] = 5 μM in 20 mM Tris-HCl buffer (pH 7.5) containing 100 mM NaCl and 0.0 (●), 2.0 (■), 4.0 (○), or 6.0 M (△) urea; rate: 1 °C min^{-1}. d) Comparison of the temperature dependences of the $[\theta]_{222}$ values of AM2 (○) and AM2W (■) in the presence of 4 M urea; [protein] = 5 μM in 20 mM Tris-HCl buffer (pH 7.5) containing 4 M urea and 100 mM NaCl; rate: 1 °C min^{-1}. Tris-HCl: tris(hydroxymethyl)aminomethane hydrochloride; $[\theta]$: mean residue ellipticity.

analysis. Samples at 4 different concentrations of AM2 and AM2W (5, 7.5, 10, and 15 μM) were analyzed at 3 different angular velocities (20000, 16000, and 10000 rpm) and the absorbance at 230 nm was observed to determine the protein-density gradients. The data for 15 μM AM2W are shown in Figure 3a, and the linear fitting of the $10^5/M_{app}$ values for each concentration of AM2W is shown in Figure 3b. Following an increment in the sample concentration, the averaged molar mass determined from the distribution data increased, which suggests that intermolecular aggregates might exist at higher concentrations. However, at least in the range below 50 μM, intermolecular species of AM2W should be less than 5%. Extrapolation of the $10^5/M_{app}$ values to determine that at 0 M gave the averaged molecular masses of AM2 and AM2W as 15019 and 14904, respectively. The data suggest that these peptides exist in solitary dispersed forms.

1-Anilino-8-naphthalene sulfonic acid (ANS) is an effective microenvironmental probe that can be used to measure the degree of hydrophobicity around a site, and it can be used to measure the folding of a protein. If a designed protein is in a labile globular state, ANS binds and produces strong fluorescence, accompanied by a blueshift of the λ_{max} value of the fluorescence.[17] The fluorescence spectra of ANS in the absence and presence of AM2 and AM2W were measured, with excitation at 350 nm. The addition of both AM2 and AM2W induced weak fluorescence increases below 515 nm; this indicates a tightly folded tetrameric structure.

AM2W has a Trp residue at the *d* position of the second heptad repeat of the third helical peptide, which is assumed to be in or next to the designed cavity. The fluorescence spectra in both the native and denatured conditions, measured with excitation at 278 nm at 30 °C, are shown in Figure 4. The Trp residue is a fluorescent chromophore, and the λ_{max} value of the fluorescence emission is 327–332 nm in the hydrophobic region and 354 nm at the aqueous surface.[18] The λ_{max} value of the Trp fluorescence of AM2W in the native state was observed at 338 nm, whereas it shifted to 348 nm after complete denaturation of AM2W with 7.0 M urea. This suggests that, in the native state, the indole ring of the Trp residue of AM2W is more dehydrated, that is, it exists at the interface of hydrophobic and hydrophilic areas or, more likely, in the loose hydrophobic cavity. This would make it a good probe to detect the binding of ligand molecules in the designed cavity of AM2W.

Binding analyses of organic ligands for AM2 and AM2W: A number of studies concerning organic-ligand binding in aqueous media were carried out by using cyclodextrin.[19] Cyclodextrin is a cyclic oligosaccharide, and it may have cavities of different sizes in its barrel-shaped form. The size of a cavity is correlated with the number of D-glucose units, connected by ether bonds between the 1- and 4′-carbon atoms, and various organic molecules can be captured in the

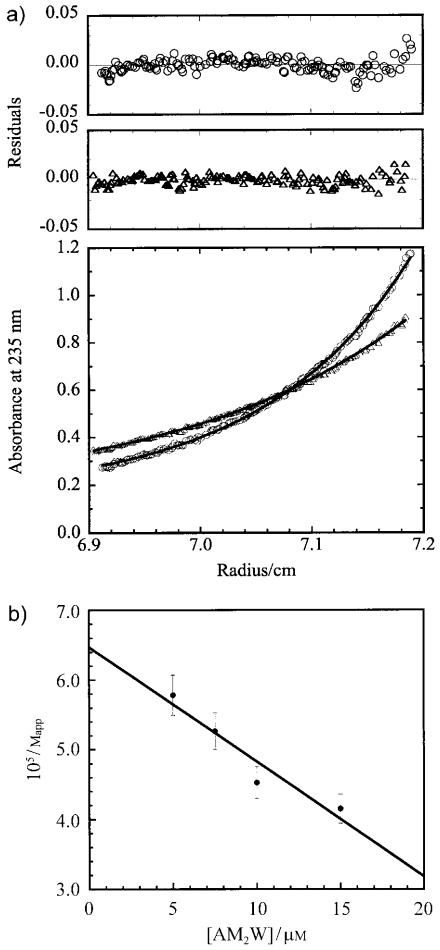


Figure 3. a) Sedimentation equilibrium data of AM2W (15 μM) at an angular velocity of 16000 rpm (△) and 20000 rpm (○). b) Determination of the buoyant molecular weight of AM2W by linear extrapolation of M_{app} (apparent molecular weight) values at each concentration.

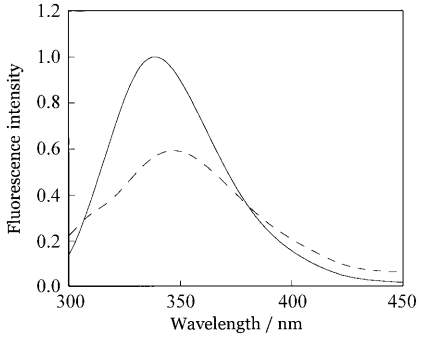
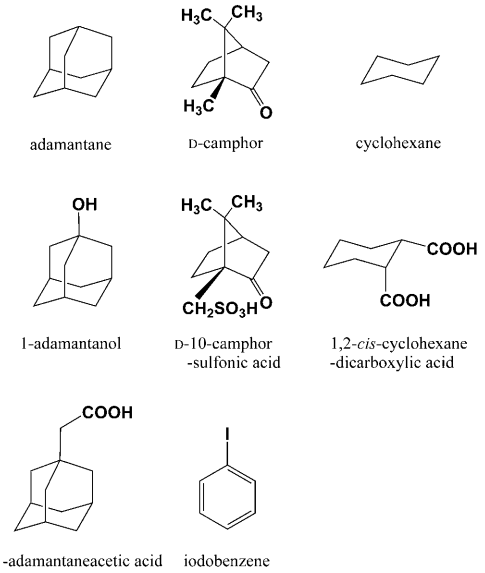


Figure 4. Fluorescence spectra of AM2W in the presence (-----) and absence (——) of 7M urea: [AM2W] = 1 μM in 20 mM Tris-HCl buffer (pH 7.5) containing 100 mM NaCl at 30°C.

hydrophobic barrel. α-Cyclodextrin has six D-glucoses, whereas β-cyclodextrin has seven and γ-cyclodextrin has eight. In the light of research by Yadav et al., the size of the cavities in our designed AM2 and AM2W proteins was predicted to be at least 8 nm in diameter,[10] if the formation of a cubic cavity is assumed, and this is comparable to that of γ-cyclodextrin. γ-Cyclodextrin has good affinities for hydrocarbon-rich molecules, such as adamantane and D-camphor derivatives. Larger organic compounds, such as retinoic acid, vitamin D3, or stearic acid, bind to this cavity in a threaded manner. Hence, for the organic ligands to be tested (Scheme 1), we chose adamantane, D- and L-camphor, 1-adamantanol, 1-adamantaneacetic acid, and D-10-camphorsulfonic acid, with consideration of their sizes and hydrophobicities. Iodobezene was also tested because it can be incorporated into the hydrophobic cavity formed with the four Ala substitutions of the tetrameric coiled coil. The smaller molecules cyclohexane and 1,2-*cis*-cyclohexane-dicarboxylic acid were also examined as negative controls.



Scheme 1. Structures of ligand molecules.

Due to the lack of a fluorescence chromophore in AM2, we used ITC measurements to assess the ligand-binding properties of AM2. A solution of 20 μM AM2 in 20 mM Tris-HCl (pH 7.5) containing 100 mM NaCl was titrated, with successive addition of each ligand solution; the heat flux was then observed. However, heat fluxes were not observed for all of the tested ligands, probably because of lower affinities due to flattening of the area around the cavity or due to other disruptions of the designed cavity structure.

AM2W has a Trp residue in or next to the designed cavity so there is a suitable fluorescence chromophore to detect ligand binding. Upon addition of D-camphor, the fluorescence of Trp decreased significantly, and the protein was saturated by addition of 100 μM D-camphor (Figure 5). Assuming 1:1 complexation, we determined the dissociation constant, K_d, from these titration data by nonlinear curve-fitting analysis and it was 23.1 μM at 30°C (Figure 5). By contrast, the binding of adamantane induced both a decrease in and a

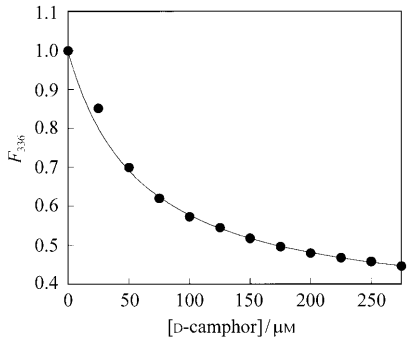


Figure 5. Changes in the fluorescence intensity at 336 nm (F_{336}) following complexation of AM2W with D-camphor: [AM2W] = 1 μM and [D-camphor] = 0–300 μM in 20 mM Tris-HCl buffer (pH 7.5) containing 100 mM NaCl at 30 °C.

small blueshift of Trp fluorescence. The single isosbestic point following the complexation of adamantane suggested the formation of a 1:1 complex (Figure 6). From a similar analysis of the titration data, we estimated the K_d value to be 220 nM at 30 °C. The other ligands, depicted in Scheme 1,

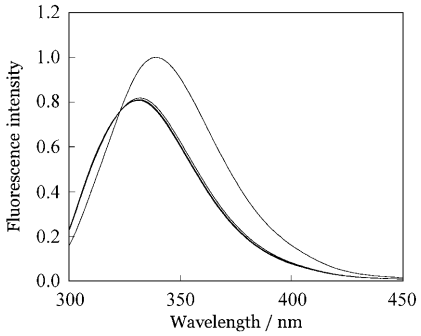


Figure 6. Fluorescence spectral changes of AM2W upon binding with adamantane: [AM2W] = 1 μM and [adamantane] = 0–5 μM in 20 mM Tris-HCl buffer (pH 7.5)containing 100 mM NaCl at 30 °C.

were also examined, and the obtained data are summarized in Table 1. Adamantane, which is the most hydrophobic and hydrocarbon-rich compound tested in our research, had the highest affinity of 220 nM at 30 °C. By contrast, 1-adamantanol, an alcohol derivative of it, exhibited a lower affinity of 81 μM, which is approximately 350-fold weaker than that of adamantane. The anionic derivative, 1-adamantaneacetic acid, has much a lower affinity of 294 μM, which is more than 10^3-fold weaker. These data suggest that the ligand binding to AM2W is mainly preceded by hydrophobic interactions with inclusion. Similar tendencies were observed for D-camphor (23.1 μM) and the charged derivative D-10-camphorsulfonic acid (< 10 mM). We also examined the binding characteristics of cyclohexane and 1,2-cyclohexanedicarboxylic acid, but their bindings were substantially weaker (< 10 mM) and multistep binding processes were simultaneously observed. Similar tendencies were also observed for bromobenzene and chlorobenzene, which are smaller benzene derivatives than iodobenzene (K_d = 5.0 μM at 30 °C); thus, size complementarity, especially for smaller ligands, is reflected in the affinity. We compared the binding affinities of D- and L-camphor because chiral discrimination of ligand molecules by AM2W was expected,. However, the ratio of binding affinities (D/L) was only 1.6, which was similar to that of cyclodextrin.[19]

Table 1. Summary of the dissociation constants (K_d in mol L^{-1}) of AM2W in binding with various organic ligands.

Ligand	AM2W	
	K_d at 30 °C [10^6 mol L^{-1}]	K_d at 20 °C [10^6 mol L^{-1}]
adamantane	0.220	0.286
1-adamantanol	81.0	109
1-adamantaneacetic acid	294	1360
D-camphor	23.1	26.1
L-camphor	14.1	20.0
D-10-camphorsulfonic acid	< 100000	< 100000
cyclohexane	< 100000[a]	< 100000[a]
1,2-*cis*-dicyclohexanedicarboxylic acid	< 100000[a]	< 100000[a]
iodobenzene	5.00	5.88

[a] Due to the formation of complicated species (1:2 or 1:3) between AM2W and these ligands, we could not determine the K_d values for the 1:1 complex.

To determine the thermodynamic parameters more precisely, we carried out isothermal titration calorimetry (ITC) measurements. We took ITC measurements only for D-camphor and D-10-camphorsulfonic acid because of the solubility of the ligands.

The dissociation constants and the thermodynamic parameters at different temperatures are listed in Table 2. The thermodynamic parameters were determined by curve-fitting analyses with the theoretical equation for 1:1 binding, as reported by Wiseman et al.[20]

Table 2. Thermodynamic parameters of AM2W in binding with D-camphor.[a]

T [°C]	K_d [μM]	ΔH[b] [kJ mol^{-1}]	$\Delta S°$[b] [J mol^{-1} °C^{-1}]	ΔC_p[b] [J mol^{-1} °C^{-1}]
14.9	25.7(±2.3)	17.4(±0.65)	148	
19.7	40.5(±3.6)	15.3(±0.66)	136	-733
25.0	25.7(±11.5)	9.91(±0.68)	121	
30.3	34.0(±6.1)	6.63(±0.58)	108	

[a] Standard deviations are based upon fitting errors. In the fitting, the binding stoichiometry was fixed to one. [b] ΔH: Energy flux; $\Delta S°$: entropy change; ΔC_p: heat capacity change.

The dissociation constants for D-camphor estimated from the ITC measurements (Figure 7) were comparable to those from the fluorescence titrations. The thermodynamic parameters with positive ΔH and $\Delta S°$ values indicate that the complexation is entropy driven. In addition, the favorable entropic contribution suggests that there are hydrophobic interactions of the hydrophobic pore with D-camphor and/or dehydration effects of D-camphor after it is captured in the pore. The negative ΔC_p value also supports the involvement of dehydration in the complexation. By contrast, the addition of D-10-camphorsulfonic acid to AM2W did not induce any detectable heat fluxes, and this reflected the lower affinity (< 10 mM).

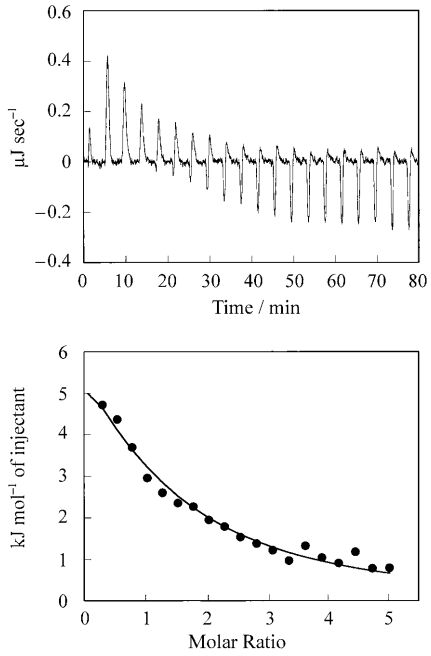


Figure 7. Isothermal calorimetry measurements for AM2W in binding with D-camphor: Top) Energy flux following injection of 500 μM D-camphor solution into a 20 μM AM2W solution at 19.7°C; bottom) plot of ΔH versus the mole ratio of added D-camphor.

Effect of ligand binding on protein stability: Ligand binding to proteins sometimes increases the protein-structure stability. However, detailed discussions and examinations of such correlations have so far been limited. As an example involving a designed protein, Alber and co-workers reported the binding of benzene in a GCN4-based trimeric coiled-coil peptide with three Ala substitutions in the hydrophobic core.[11] They demonstrated an incremental increase in the structure stability by benzene binding to the designed hydrophobic pore formed with the Ala substitutions. In our work, we examined the same kind of effect of ligand binding on the structure stability of AM2W by measuring the T_m value. We used adamantane, 1-adamantanol, and 1,2-*cis*-cyclohexanedicarboxylic acid as the selected ligands. The binding-affinity preference of AM2W is adamantane (220 nM) > 1-adamantanol (81 μM) > 1,2-*cis*-cyclohexanedicarboxylic acid (> 10 mM), as shown in Table 1.

We observed AM2W in the presence of urea to lower the T_m value because the T_m value of AM2W under conditions without urea as a denaturing agent is above 95°C. In the presence of 4 M urea, the T_m value of AM2W was observed to be 76°C (Figure 2d). Each temperature-dependent profile of the $[\theta]_{222}$ value before and after the addition of ligands was measured in the presence of 1% acetonitrile because the addition of adamantane was performed by the addition of an acetonitrile solution of adamantane. After the addition of 200 μM adamantane to AM2W, the T_m value increased to 81°C, with an accompanying decrease in the $[\theta]_{222}$ value (Figure 8). In the presence of 4 M urea, the binding constant of AM2W with adamantane would be expected to be less than 10 μM. Therefore, an effective concentration

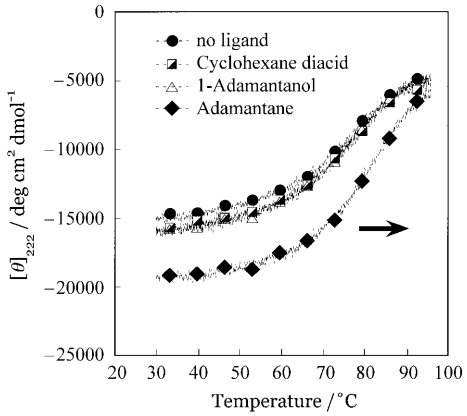


Figure 8. Temperature dependence of the $[\theta]_{222}$ value before and after addition of various ligands: [AM2W] = 5 μM and [ligand] = 200 μM in 20 mM Tris-HCl buffer (pH 7.5) containing 4 M urea, 1% acetonitrile, and 100 mM NaCl; rate: 1°C min^{-1}.

of adamantane for protein stabilization would be reasonable. By contrast, the other ligands did not exhibit increased T_m values, due to lower affinities. Addition of adamantane raises the helicity by approximately 26% at 30°C, which indicates that the binding of adamantane repaired the disruptions in the helical structure around the ligand-binding site.

The thermal-denaturation characteristics of single-chained coiled-coil proteins in the presence of guanidine hydrochloride have been examined by Bryson et al.[15c] In their work on this subject, they have observed a concomitance of low- and high-temperature denaturation behaviors in the presence of higher concentrations of denaturing agents. However, in our method, denaturing behavior was not observed at cooler temperatures, in the range of 20 to 95°C, in the presence of 4 M urea. Therefore, the decrease in the $[\theta]_{222}$ values in the lower temperature range (20–60°C) by adamantane may have been induced by its binding, due to the complementation of the molecule resulting in structure stabilization.

Discussion

To encourage the study of de novo designed coiled-coil proteins for the creation of new receptors or enzymes, a broadening of design concepts relating to the ligand-binding site would be helpful. In our experiments, we examined the binding of ligand molecules with various sizes and hydrophobicities in designed hydrophobic cavities in a four-unit coiled-coil protein by using fluorescence and ITC measurements. Ala substitutions at neighboring *a*- and *d*-positions in the bundle structure allowed the creation of a cavity in the hydrophobic core of the de novo designed structure. However, excessive Ala substitutions did not allow the formation of a stable cavity and, therefore, AM2 did not exhibit any ligand-binding affinity. Two Ala substitutions in the hydrophobic region of a three-helical-bundle peptide constructed on a template peptide were examined by Obataya et al., and

they concluded that the two substitutions of hydrophobic residues resulted in critical destabilization of the bundle structure.[21] Our data on AM2 are consistent with this. On the other hand, a combination with a Trp substitution in the designed cavity resulted not only in an increase in protein-structure stability but also in avoidance of pocket-structure flattening out; this differs from the case of AM2. As a result, AM2W exhibited clear ligand-binding affinity. At the ligand-binding site in natural and designed proteins, some key residues sometimes take on important roles in maintaining an active concavity or cleft structure in the apo state.[22] In this experiment, Trp substitution was effective in forming the ligand-binding site in the coiled-coil protein.

The size-complemented ligands, such as adamantane and D-camphor, have higher affinities for AM2W than those of noncorresponding size. The smaller ligands, such as cyclohexane, exhibited significantly lower affinities, approximately in the micromolar range. However, because the size selectivity of AM2W for ligand molecules is stringent, AM2W did not exhibit chiral discrimination abilities. Hydrophilic groups in the ligand molecules significantly decrease the binding affinity to AM2W, inspite of the existence of acceptors for hydrophilic interactions at the *e* and *g* positions in the protein. To further improve the design of selective ligand binding, the introduction of hydrogen bonds, ion-pair interactions, etc. in the hydrophobic core will be necessary.

The size of the hydrophobic cavity of AM2W is expected to be similar to that of γ-cyclodextrin. Therefore, we analyzed the binding affinities with ligand molecules that are commonly used in cyclodextrin chemistry. The ΔH and $\Delta S°$ values of γ-cyclodextrin and 1-adamantaneacetic acid in binding at 25 °C were reported to be 7.5 kJ mol^{-1} and 83.6 J mol^{-1}, respectively,[23] whereas we obtained ΔH and ΔS values of 9.91 kJ mol^{-1} and 121 J mol^{-1}, respectively, in the complexation of AM2W with D-camphor at 25 °C. These tendencies seem to be similar. By contrast, the enthalpy–entropy compensation phenomenon is known to occur in the complex formation of cyclodextrin with hydrophobic ligands. In Figure 9, we show the ΔH versus $T\Delta S°$ plot of AM2W binding with D-camphor. Interestingly, the plot seems to be linear, and its slope is 0.86, which is typical in cyclodextrin systems.[18] The good linearity in the ΔH versus temperature plot for estimating the ΔC_p value also supports the idea that there is a single process of interaction in the binding with D-camphor. The binding of D-camphor did not result in significant effects on the CD spectrum of AM2W so the obtained thermodynamic parameters in Table 2 may be attributed to interactions between the designed hydrophobic pore and D-camphor. In the complexation of D-camphor and AM2W, the ΔC_p value was negative, which suggests a contribution by both large dehydration effects of hydrophobic ligands and hydrophobic interactions.

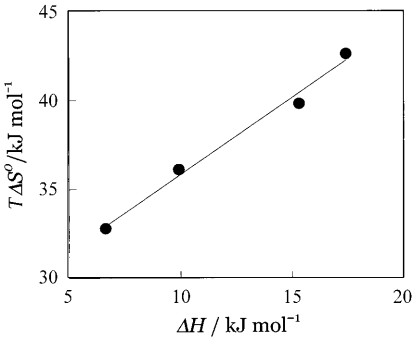


Figure 9. Enthalpy–entropy compensation plot for AM2W in binding with D-camphor.

Conclusion

We designed tetrameric coiled-coil proteins with a hydrophobic cavity at the hydrophobic core. From fluorescence and ITC measurements, we determined the dissociation constants of AM2W for various organic ligands. The ligand molecules with higher affinity for the hydrophobic cavity induced an apparent stabilization effect in protein structure. These findings will be valuable in the design of de novo receptor and enzyme proteins in the future.

Experimental Section

Taq DNA polymerase, restriction enzymes, T4 DNA ligase, and alkaline phosphatase were purchased from Takara Shuzo, Japan. Common chemicals, isopropyl-β-D-thiogalactopyranoside (IPTG), bacto tryptone, agar, and yeast extract (Wako Chemicals, Japan), ampicillin (Meiji Seika, Japan), Agarose ME (Iwai Chemicals, Japan), and glycogen (Roche Diagnostics, Japan) were used without further purification.

Expression and purification of tetrameric coiled-coil variant proteins: The DNA fragments, with NcoI and HindIII restriction enzyme sites at each end, for the genes of AM2 and AM2W,were inserted into a pET-32a(+) plasmid vector (Novagen). An *Escherichia coli* BL21(DE3) strain harboring these plasmids was cultured at 37 °C for 5 h in Luria–Bertani medium (250 mL) and then at 37 °C for 3 h in the presence of 1 mM IPTG. The cells were harvested, resuspended in 20 mM Tris-HCl buffer (7 mL; pH 7.5), and sonicated. The suspension was centrifuged, and the supernatant fraction was separated by decantation. The supernatant fraction was exposed to His-Bind Resin (Novagen; 5 mL). After application of the expressed protein solution to the resin, the resin was washed with 20 mM Tris-HCl buffer (30 mL; pH 7.9) containing 500 mM NaCl and 5 mM imidazole and then again with the same type of buffer (50 mL) containing 500 mM NaCl and 60 mM imidazole. The target protein was eluted with the same type of buffer containing 1 M imidazole (20 mL), and each fraction was analyzed by using 15% sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE). The fractions containing the target protein were collected and dialyzed three times against 20 mM Tris-HCl buffer (2 L; pH 8.4) containing 150 mM NaCl and 2.5 mM $CaCl_2$ at 4 °C. This dialyzed solution was used in thrombin digestion to remove the thioredoxin fusion protein from the AM2 and AM2W proteins. Finally, the mixtures were used in reversed-phased HPLC separation, and the AM2 and AM2W proteins were isolated. Refolding was carried out by slow dilution of the denaturing agent guanidine hydrochloride in the 6 M guanidine hydrochloride solution of each protein. The concentration of each protein solution was determined from the absorbance at 280 nm (A_{280}) in 6 M guanidine hydrochloride solutions by comparing with the molar coefficients (ε_{280} = 5120 M^{-1}cm^{-1} for AM2 and 10810 M^{-1}cm^{-1} for AM2W).[24]

Circular dichroism (CD) measurement: All CD measurements were carried out by using a JASCO J-820 spectrometer with a 2 mm pathlength

cuvette. The CD spectra of AM2 and AM2W (5 μM) were measured in 20 mM Tris-HCl (pH 7.5) containing 100 mM NaCl. The [θ] values are given in units of deg $cm^2 dmol^{-1}$.

Fluorescence measurements of AM2W in the presence and absence of ligands: Fluorescence measurements were performed by using a JASCO FP-6200 fluorescence spectrophotometer with a thermocontroller apparatus and a 1 cm pathlength cuvette. The emission spectra of Trp from 300 to 450 nm were measured with excitation at 278 nm. The measurements were performed in 20 mM Tris-HCl (pH 7.5) containing 100 mM NaCl at 20 and 30 °C. The protein concentration was 0.1 or 1.0 μM.

Thermal-denaturation experiments in the presence and absence of ligand: The thermal-denaturation curve, which was obtained by monitoring the $[\theta]_{222}$ values and averaging 10×1.0 s readings, was obtained with a 5 μM protein solution in 20 mM Tris-HCl (pH 7.5) containing 100 mM NaCl and 0–7.5 M urea. In the case of ligand addition, the concentration of ligand was 200 μM.

Analytical ultracentrifugation experiment: To determine the molecular weights of AM2 and AM2W, the sedimentation equilibrium method was used. 4 different concentrations (5, 10, 15, 20 μM) of AM2 and AM2W solutions in 20 mM Tris-HCl buffer (pH 7.5) containing 100 mM NaCl were separately placed in analytical cells with double-sector centerpieces. Sedimentation equilibrium experiments were performed by using a Beckman Optima XL-I analytical ultracentrifuge with double-sector centerpieces and sapphire windows, at 16000, 20000, and 24000 rpm at 30 °C. Absorbance scans at 230 nm were measured in radial steps at 0.001 cm intervals, and data were collected by taking the average of 16 measurements at each radial distance. The approach to equilibrium was considered to be complete when replicate scans that were separated by 6 h were indistinguishable. Their partial specific volumes (0.751 $cm^3 g^{-1}$ for AM2 and AM2W) were estimated from the amino acid composition by the method of Cohn and Edsall.[25] The density of the solution was assumed to be 1.00 $g cm^{-3}$. Each piece of data obtained was analyzed by using the Origin software (Version 6.0.4) supplied by the Beckman Co., Ltd., and the molecular weights of AM2 and AM2W were estimated by global fitting analysis.

Isothermal titration calorimetry measurements: ITC experiments were carried out by using a MicroCal MCS calorimeter interfaced with a microcomputer. All samples were in 20 mM Tris-HCl buffer (pH 7.5) containing 100 mM NaCl, and all solutions were thoroughly degassed by using the degassing equipment provided with the instrument. The ligand solution was titrated into the AM2 and AM2W solutions (20 μM). Each titration consisted of a preliminary 2 μL injection followed by 23 subsequent 5 μL additions, which were performed over 10 s periods at 240 s intervals. The heat for each injection was subtracted from the dilution heat of the titrant, and each heat correction was divided by the number of moles of ligand injected. Data were analyzed by using the Origin software supplied by MicroCal.

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Received: May 6, 2008
Revised: November 13, 2008
Published online: December 29, 2008

