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Hapten-Induced Dimerization of a Single-Domain VHH Camelid Antibody[†]

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ABSTRACT: Antibodies that recognize small molecule ligands (haptens) provide unique insight into the immune response and frequently serve as biological reagents for the detection of small molecules. While conventional antibodies typically recognize haptens using two variable domains (VL and VH), much less is known regarding how antibodies with a single variable domain recognize small ligands. Here we investigate the binding thermodynamics for an anti-caffeine camelid (VHH) antibody. Surprisingly, a nonconventional binding stoichiometry was observed in which the final complex includes two VHH domains for every caffeine molecule. ΔC_p analysis and size exclusion chromatography support this unusual stoichiometry. An apparent consequence of ligand-induced dimerization is that a relatively high affinity ($K_{b,obs} = 7.1 \times 10^7$) is obtained. The binding profiles of three caffeine metabolites, theophylline, theobromine, and paraxanthine, were also investigated. Each ligand maintains a 2:1 stoichiometry while displaying an ~50-fold range of observed binding affinities. These results suggest nonconventional mechanisms of hapten recognition are possible with single-domain antibodies.

The interaction between antibodies and low-molecular mass molecules, termed haptens, are of interest for a variety of applications, such as diagnostics and chromatography. Traditionally, anti-hapten antibodies are produced in vivo by injection of the small molecule which has been covalently "tethered" to a larger protein to elicit an immune response. A wide range of different hapten molecules, such as dyes, hormones, pesticides, and antibiotics, have all been used to generate antibodies that recognize their respective small molecule target with high affinity and specificity (*1*). Since the resulting IgG antibodies are relatively large in size (150 kDa), fragments of these antibodies, such as Fab (~60 kDa) and scFv (~30 kDa), are frequently engineered. These reduced antibody fragments maintain the necessary ligand binding domains (i.e., the variable domains).

The discovery of a class of camelid antibodies (including camels and llamas) that lack light chains has provided a biological example in which antigen recognition is reduced to a single domain (*2*). Despite possessing an interface with only three complementarity-determining regions (CDRs), camelid antibodies, termed VHH, can bind protein antigens with affinities comparable to those of their larger antibody counterparts (*3*). However, much less is known regarding their ability to recognize small molecule haptens.

VHH domains have been raised against protein−hapten conjugates such as azo dye RR120 (*4*), methotrexate (MTX) (*5*), the herbicide picloram (*6*), and the myotoxin 15-acetyldeoxynivalenol (*7*). In general, these VHH domains possess K_d values in the micromolar range as assessed through immunological-based methods. While these examples demonstrate the plausibility of small molecule recognition by a single-domain VHH, a thermodynamic understanding of binding has been lacking. Furthermore, structural data of VHH−hapten complexes are rather limited. Two complexes between VHH and azo dyes RR6 (*8*) and RR1 (*4*) have been reported which reveal the formation of a crevice between CDR3 and CDR2.

Recently, Ladenson and co-workers generated an anti-caffeine VHH antibody through a combination of llama immunization and phage display techniques (*9*). Caffeine, a central nervous system stimulant, represents a relatively simple ligand because of its small size (194 Da). Consequently, the anti-caffeine VHH serves as a useful model for the investigation of small molecule recognition by single-domain antibodies. Here we investigate the biophysical properties of anti-caffeine antibody−caffeine recognition.

Starting with a gene for an anti-RNase A VHH antibody (kindly provided by S. Koide, University of Chicago, Chicago, IL) (*10*), we used Kunkel mutagenesis (*11*) was used to replace the three CDR loops with those of the anti-caffeine VHH antibody (*9*). The anti-caffeine VHH was then expressed in *Escherichia coli* and purified (Supporting Information).

To investigate the biophysical properties of caffeine−anti-caffeine VHH binding, isothermal titration calorimetry (ITC) was performed (Supporting Information) to provide a full thermodynamic profile of binding (K_b, $\Delta G°$, $\Delta H°$, and $\Delta S°$). Experiments at 25 °C illustrated that binding is dominated by a favorable enthalpy ($\Delta H° = -14$ kcal/mol), which overcomes a small entopic penalty ($-T\Delta S° = 3.9$ kcal/mol), leading to an overall $\Delta G°$ of −10 kcal/mol ($K_{b,obs} = 7.1 \times 10^7$). The observed K_b is surprisingly large considering it is a hapten−single-domain antibody complex. However, the most striking feature was the observed binding stoichiometry, which was determined to be 0.5 (Figure 1), suggesting a 2:1 anti-caffeine VHH:caffeine binding stoichiometry.

The binding interfaces of hapten−antibody complexes are typically found (or simply assumed) to conform to a 1:1 binding

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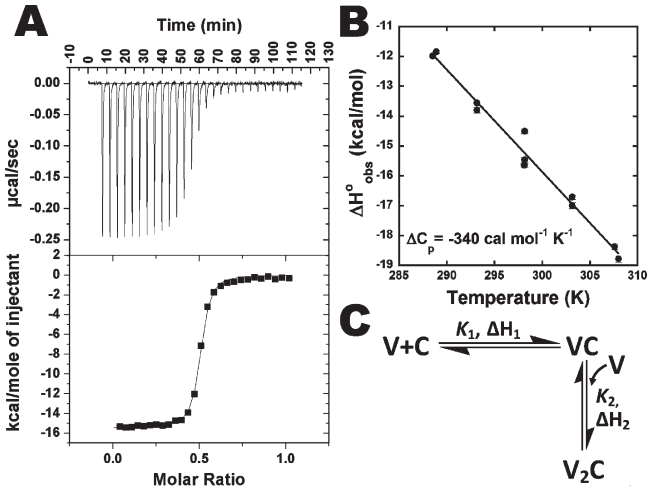

FIGURE 1: (A) Isothermal titration between caffeine and the anti-caffeine VHH. (B) Plot of ΔH°_{obs} as a function of temperature for VHH−caffeine binding. (C) Thermodynamic model of caffeine-induced dimerization (V, VHH; C, caffeine).

ratio, where one hapten binds a single VH−VL (or VH) interface. This assumption is generally reasonable considering the large size difference between the hapten and the VH−VL interface. While a recent structural and biophysical analysis of a 772 Da tetrasulfocyanine dye−anti-dye Fab complex demonstrated a 2:1 binding stoichiometry is possible (*12*), the 2:1 interaction was mediated through dye symmetry. Here, caffeine is much smaller and lacks such symmetry.

To further explore the nature of the intermolecular interactions, the change in heat capacity (ΔC_p) upon caffeine−VHH binding was determined by examining the temperature dependence of the observed binding enthalpies over a temperature range from 15 to 35 °C (Figure 1B). Changes in heat capacity upon binding can be a useful gauge of the amount of surface area that is buried upon protein folding, protein−protein binding, or protein−ligand binding (*13*) and/or serve as an effective indicator of the presence of a linked equilibrium, such as ion binding (*14*) and proton linkage (*15*). The observed ΔC_p upon caffeine−antibody binding was -340 ± 20 cal mol^{-1} K^{-1}. While a negative ΔC_p is quite common for both protein−protein (*16*) and protein−ligand interactions (*17*, *18*), here the observed ΔC_p is quite large in magnitude considering the interaction is between a small molecule ligand and protein, which is typically in the range of -100 to -200 cal mol^{-1} K^{-1} for molecules the size of caffeine (*17*, *18*).

Structure-based thermodynamic calculations provide an approach to estimating the average ΔC_p contribution per unit surface area (*19*). Caffeine possesses a surface area of 350 Å^2. Assuming it is completely buried in a 1:1 complex with the antibody, approximately 700 Å^2 of surface area may be buried. This amount of surface area burial would equate to a ΔC_p of approximately -100 cal mol^{-1} K^{-1} [using 0.15 cal K^{-1} mol^{-1} Å^2 (*19*)]. This calculated value is in the range of what is expected for a typical small molecule−protein complex. The observed ΔC_p of -340 ± 20 cal mol^{-1} K^{-1} would require approximately 3-fold more surface burial. Such surface area burial may be possible with a 1:2 binding stoichiometry, where in addition to caffeine burial, a significant amount of surface area would be buried due to VHH−VHH contacts.

VHH oligomerization was examined using size exclusion chromatography. VHH with or without caffeine was mixed and run over a Superdex-75 column (Figure 2). Free anti-caffeine VHH (concentrations of up to 1.4 mM which are 140 times that used in ITC experiments) displayed a peak that overlaps with that of the original anti-RNaseA VHH antibody (13.1 kDa) (data not shown). With increasing concentrations of caffeine, a higher-molecular mass peak appears, which is consistent with a VHH dimer (~26.4 kDa), based on a molecular mass ladder (Supporting Information). Molar ratios of 2:1 (anti-caffeine VHH:caffeine) were sufficient to generate almost 100% VHH dimer. These results are consistent with the stoichiometry determined from ITC, suggesting the presence of an unconventional 2:1 binding stoichiometry for an anti-hapten camelid VHH antibody (Figure 1C).

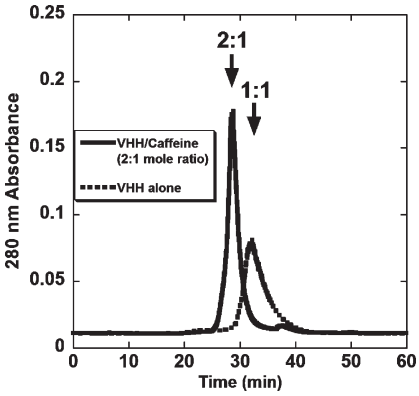

FIGURE 2: Gel filtration analysis of the anti-caffeine VHH in the absence and presence of stoichiometric molar concentrations of caffeine.

Table 1: Binding Thermodynamics for Caffeine Metabolites[a]

Ligand	ΔG°_{obs} (kcal/mol)	ΔH°_{obs} (kcal/mol)	$-T\Delta S^{\circ}_{obs}$ (kcal/mol)	K_b (x 10^7)	N
Caffeine	-10.7 ±0.1	-15.0 ±0.1	4.3 ±0.1	7.1 ±0.9	0.496 ±0.002
Theophylline	-9.56 ±0.06	-13.4 ±0.1	3.81 ±0.1	0.99 ±0.09	0.516 ±0.004
Paraxanthine	-8.53 ±0.05	-9.9 ±0.2	1.37 ±0.2	0.18 ±0.01	0.500 ±0.006
Theobromine	-8.44 ±0.03	-12.4 ±0.1	3.96 ±0.1	0.15 ±0.01	0.478 ±0.002

[a] ITC experiments were performed at 25 °C in 20 mM sodium phosphate buffer and 150 mM sodium chloride.

To help reveal the structural basis of recognition, and potentially dimerization, we examined the binding thermodynamics for three caffeine metabolites, theophylline, paraxanthine, and theobromine (Table 1). Each analogue is structurally similar to caffeine, with the exception that each is missing a single methyl group. The four ligands displayed a 50-fold (~2 kcal/mol) range of binding constants while maintaining the 2:1 binding stoichiometry. Each possesses similar binding thermodynamics, where binding is enthalpically driven, overcoming a small entropic penalty. Theophylline displays the binding profile most similar to that of caffeine. This is likely explained by the fact that theophylline differs in the absence of the N7 methyl group. This position was used for protein conjugation during the original immunization (*9*) and would therefore be less likely to participate in energetically critical contacts with VHH. On the other hand,

paraxanthine and theobromine both display an ~2 kcal/mol decrease in affinity upon loss of the N3 and N1 methyl groups, respectively. As these two methyl groups are located on the opposite side of the N7 conjugation site, they are likely to be buried in the initial VHH−caffeine complex and appear to play a significant role in their interactions with the antibody as the loss of either methyl group adversely affects the observed affinity, although a 2:1 stoichiometry is maintained.

The 2:1 binding stoichiometry between VHH and caffeine represents an atypical example of how small molecules are recognized by antibodies. In general, the reduced interface of a single-domain antibody may be considered a limitation toward small molecule recognition. In fact, small molecule binding sites in conventional antibodies frequently occur at the VL−VH interface (*20*, *21*), which is absent with only a single VHH domain. The results presented here provide evidence that high-affinity interactions may be possible, albeit through ligand-induced VHH dimerization. Consequently, the observed affinity for caffeine is roughly 1000-fold higher than estimates based on the original enzyme-linked immunosorbent assay study (*9*).

A model consistent with the apparent differences in observed affinity includes the stepwise formation of the 1:1 and 2:1 VHH−/caffeine complexes (Figure 1C). Consequently, $K_{b,obs}$ is equal to $K_1(1 + K_2[VHH])$, where K_1 and K_2 are the equilibrium constants for formation of the 1:1 and 2:1 VHH−caffeine complexes, respectively. Binding assessed as a function of VHH concentration should allow determination of the two microscopic equilibrium constants, although the VHH concentration dependence was not explored here because of the high ligand concentrations required for calorimetry. However, as we were unable to detect a calorimetric signal for multisite or cooperative binding, which included performing reverse titrations (Supporting Information), or to detect any evidence of VHH dimerization in the absence of caffeine, our data thus far suggest that K_2 (VHH "dimerization") is much greater than K_1 (caffeine binding).

To fully decipher the mechanism of this unusual 2:1 stoichiometry, an understanding of the molecular details will be necessary. For the sake of comparison, a similar stoichiometry was recently elucidated for a 2:1 anti-peptide Fab fragment−myc tag complex (*22*). In this example, the nonsymmetric peptide serves to bridge two Fab fragments in a head-to-head orientation. While it is difficult to extrapolate from this example, the lack of caffeine symmetry coupled with the observation that significant surface must be buried upon binding suggests extensive VHH−VHH contacts. These VHH contacts appear to be energetically feasible only upon formation of the 1:1 VHH−caffeine complex. Thus, the 1:1 complex appears to provide a "new" binding site for the second antibody. Crystallographic investigations into the structure of the complex will help reveal the nature of these interactions and mechanism of recognition.

Overall, we present an atypical model of molecular recognition between a single-domain VHH antibody and its hapten. The 2:1 binding model provides a route toward VHH-driven high-affinity antibody−hapten binding. These results demonstrate that alternate mechanisms of recognition may be considered for single-domain antibodies, such as VHH.

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SUPPORTING INFORMATION AVAILABLE

Additional materials and methods and an ITC figure. This material is available free of charge via the Internet at http://pubs.acs.org.

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