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Dominant Alcohol–Protein Interaction via Hydration-Enabled Enthalpy-Driven Binding Mechanism

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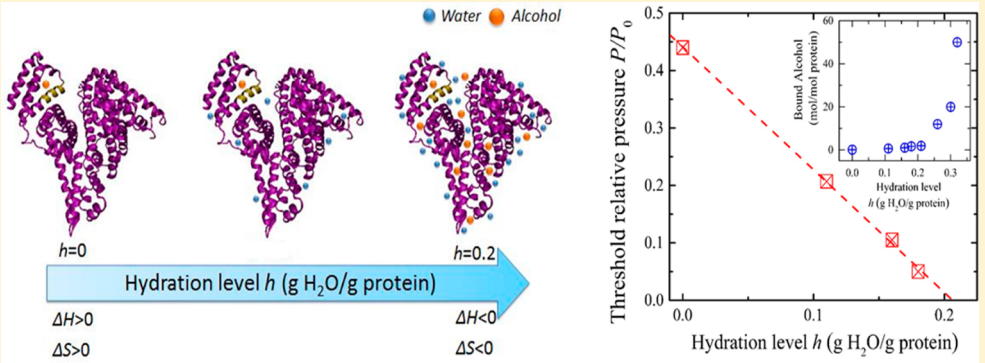
Supporting Information



ABSTRACT: Water plays an important role in weak associations of small drug molecules with proteins. Intense focus has been on binding-induced structural changes in the water network surrounding protein binding sites, especially their contributions to binding thermodynamics. However, water is also tightly coupled to protein conformations and dynamics, and so far little is known about the influence of water–protein interactions on ligand binding. Alcohols are a type of low-affinity drugs, and it remains unclear how water affects alcohol–protein interactions. Here, we present alcohol adsorption isotherms under controlled protein hydration using in situ NMR detection. As functions of hydration level, Gibbs free energy, enthalpy, and entropy of binding were determined from the temperature dependence of isotherms. Two types of alcohol binding were found. The dominant type is low-affinity nonspecific binding, which is strongly dependent on temperature and the level of hydration. At low hydration levels, this nonspecific binding only occurs above a threshold of alcohol vapor pressure. An increased hydration level reduces this threshold, with it finally disappearing at a hydration level of $h \approx 0.2$ (g water/g protein), gradually shifting alcohol binding from an entropy-driven to an enthalpy-driven process. Water at charged and polar groups on the protein surface was found to be particularly important in enabling this binding. Although further increase in hydration has smaller effects on the changes of binding enthalpy and entropy, it results in a significant negative change in Gibbs free energy due to unmatched enthalpy–entropy compensation. These results show the crucial role of water–protein interplay in alcohol binding.

INTRODUCTION

Water is known to play an important role in a wide range of molecular recognition and association processes, such as protein ligand binding.[1−7] Specifically, a large number of small molecule drugs exert their influence via binding to target proteins with low affinity. These weak association processes could be particularly influenced by water, but the details are far from understood.[7−10] Evaluating the role of water in such weak associations is a critical step in understanding the binding mechanisms and biological actions of many small molecule drugs, with important implications for drug design. Alcohols are such low-affinity drugs that affect neurological responses in various ways. For instance, it is well-known that small ethanol doses stimulate a pleasurable sensation as well as cause depressant effects such as anxiety reduction; a larger dosage produces anesthetizing effects, including unconsciousness and analgesia.[11,12] The very low binding affinity of alcohols to proteins makes it difficult to recognize and characterize bound alcohols. Hence, the mechanism governing alcohol binding remains poorly understood.[11−16]

Specifically, emerging evidence suggests that water exists around alcohol binding sites.[11−18] Although the potential importance of water in alcohol–protein interactions has been recognized, it remains unclear how water contributes to the interaction. In general, it was proposed that water can contribute to alcohol binding via two mechanisms. One proposed mechanism is that water around the binding sites is displaced by alcohols.[11−17,19] The release of ordered water

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molecules to bulk water is believed to cause a gain in entropy;[10,11,13,17,19] however, this process also reconstructs water−water interactions, providing favorable enthalpic changes.[10,20−23] The controversial effects of the displacement of water on binding thermodynamics have attracted great attention.[22] Another proposed mechanism is that water molecules remain at the binding sites and form hydrogen-bond bridges linking the protein and alcohols.[13,19] These two mechanisms are both concerned with the influence of structural modifications in the water network on binding.

In addition to these prevalent mechanisms, water may also affect alcohol binding indirectly via altering protein structures and dynamics. Hydration water is known to be an integral part of the protein, as it is tightly coupled to the protein configuration and flexibility.[3−5,24−28] Ligand binding not only modifies the structure of the water network, but could also cause protein conformation changes enabled by water−protein interactions.[29] Recent works suggest that the protein is actively involved in ligand binding and can act as a potential thermodynamic reservoir: changes in protein configuration may significantly contribute to the enthalpy and entropy of ligand binding.[29−33] In particular, such protein configurational change does not necessarily involve large protein segments; it could be subtle, such as the local structural rearrangement or fluctuation of residues near binding sites.[29] These findings indicate that it might be inappropriate to consider all of the binding sites on proteins as rigid cavities and only discuss the contribution of the water network to ligand binding, especially for those proteins with high structural and dynamic adaptability. The protein's hydration state could greatly influence how the protein responds to alcohol binding, with significant influence on binding free energy. So far, however, little is known about the importance of such protein−water interaction in alcohol binding and its functional consequences.

In protein solutions, under the influence of a large amount of bulk solvent, it is difficult to evaluate the influence of hydration on ligand binding. In particular, hydration water molecules bind at different sites on the protein such as polar groups and nonpolar groups,[34,35] and such different hydration water molecules could have very different effects on ligand binding. One way to separate the influence of different hydration water molecules in alcohol binding is to evaluate alcohol binding under controlled protein hydration. Briefly, similar hydration regimes were identified for globular proteins such as bovine serum albumin (BSA) and hen egg-white lysozyme:[34,35] when the protein hydration level h < ∼0.06 (g water/g protein), water binds to charged groups on the protein surface; when 0.06 < h < ∼0.2, water covers polar sites; when h > ∼0.2, water weakly interacts with remaining protein regions; when h > ∼0.5, bulk water exists around the protein. This suggests that by controlling protein hydration level, we might be able to investigate the distinct roles of hydration water at different protein sites on alcohol binding.

Here, we employed an in situ nuclear magnetic resonance (NMR)-based isotherm measurement, which enables us to selectively detect low affinity interacting adsorbates with high sensitivity while subjecting the protein to a controlled level of hydration. We investigated ethanol (EtOH) and trifluoroethanol (TFE) interactions with bovine serum albumin (BSA). EtOH has a wide range of physiological effects on the human body,[11,12] and TFE is a potent anesthetic.[36,37] BSA is a typical globular protein with well-known crystal structure and function and has great binding capacity; hence, it serves as a good model protein in many biological and medical studies.[38,39] This NMR-based method allows us to directly characterize the very weak alcohol−protein interaction as a function of protein hydration level. Furthermore, changes in Gibbs free energy, enthalpy, and entropy associated with alcohol binding were also determined from the temperature-dependent isotherms. We found that only a few alcohol molecules bind to several high-affinity sites in the dry protein. However, a large number of alcohols could bind at low-affinity nonspecific sites when facilitated by hydration, overwhelming the number of adsorbed alcohol at high-affinity sites. At low hydration level, such dominant nonspecific alcohol binding only occurs when the alcohol vapor pressure exceeds a threshold level. This threshold is reduced by hydration and becomes negligible at a crossover hydration level of $h \approx 0.2$ (g water/g protein). Hydration also gradually changes such binding from an entropy-driven to an enthalpy-driven process. Water molecules bound at charged and polar groups on the protein surface were found to be particularly crucial for such binding. Further hydration of the protein has smaller effects on the enthalpic and entropic changes but still results in a significant decrease in Gibbs free energy upon alcohol binding. A significant difference is recognized between the role of water in alcohol binding and its role in the binding of other general anesthetics such as halothane.[40] The present work revealed the importance of water−protein interactions in alcohol binding.

METHODS

NMR Isotherm Measurements. BSA (lyophilized powder, ≥98%, pH ≈ 7, 1% in 0.15 M NaCl) was purchased from Sigma-Aldrich and used without further purification. Ethanol (99.5%, anhydrous) and 2,2,2-trifluoroethanol (99.8%, extra pure) were purchased from Fisher Scientific. BSA was put into a quartz NMR tube, which was connected directly to an in situ vapor-loading system.[40,41] Distilled water (H_2O) and liquid alcohols were stored in source bottles with pressure buffer chambers. The vapor pressure of water or alcohols was controlled by adjusting the valves close to the buffer chambers. The temperature of the sample was controlled by regulating the temperature of the airflow surrounding the quartz NMR tube. ^{1}H and ^{19}F NMR spectra were used to determine the amount of water or alcohol sorption in the protein. The protein sample was first pumped at room temperature by a mechanical pump for 1−2 days, to remove preadsorbed water. Before exposure to alcohol or water vapors, a broad ^{1}H NMR spectrum (full width at half-maximum around 40 kHz) associated with the dry protein was measured. This spectrum was used as a quantitative reference to evaluate the amount of alcohol or water sorption in the protein.[40,41] To measure the isotherms of EtOH or TFE in dry protein, the dry BSA was exposed to EtOH or TFE vapor at a given vapor pressure P. The ^{1}H free-induction-decay (FID) signal was detected for EtOH. A sharp peak above the broad (∼40 kHz) peak was observed. The ^{1}H NMR signal of EtOH vapor in the empty NMR tube without a protein sample is negligible as compared to this sharp peak. Therefore, this sharp peak is associated with EtOH sorption in BSA. The area ratio of the sharp peak to the broad peak was converted to the molar ratio of bound EtOH to BSA, according to the molar number of protons in EtOH and BSA. Isotherms were obtained by carrying out the measurements as a function of alcohol vapor pressure P. For TFE sorption, ^{1}H NMR spectra of TFE were also used to quantify the amount of sorption, and this was then used to quantify the ^{19}F NMR intensity. To measure the

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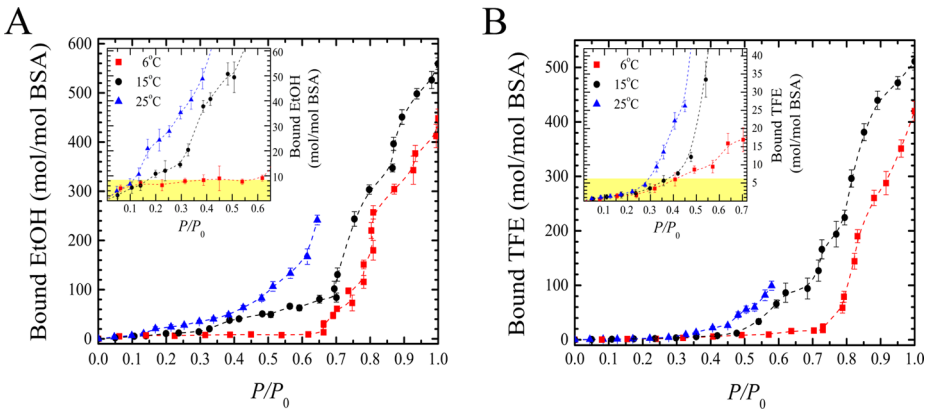

Figure 1. Isotherms of (A) EtOH and (B) TFE in dry BSA at 6, 15, and 25 °C. The insets show isotherms below $P/P_0 \approx 0.7$. Thresholds of relative vapor pressure in the isotherms are recognized. The threshold of relative vapor pressure is $P/P_0 \approx 0.15$ for EtOH (inset of A) and $P/P_0 \approx 0.3$ for TFE (inset of B). The sorption of both alcohols shows little temperature dependence below this threshold and is marked with shading in yellow. Above the threshold, alcohol sorption is enhanced by temperature. Sharp alcohol uptake above the relative vapor pressure of $P/P_0 \approx 0.7$ occurs for both alcohols, associated with protein denaturation. At each alcohol vapor pressure, NMR signal was measured five times when the interaction reached equilibrium, and then the standard deviations of NMR peak areas were used to calibrate the error bars in the isotherms.

isotherms of TFE in partially hydrated protein, the dry BSA was first exposed to the water (H_2O) vapor, and the hydration level as measured by water sorption was determined by ^{1}H NMR.[41] Thereafter, the partially hydrated BSA was exposed to TFE vapor, and ^{19}F NMR was used to measure the sorption of TFE.

Determination of ΔG, ΔH, and ΔS. These thermodynamic quantities of EtOH or TFE−protein interaction were determined from the isotherms at different temperatures.[42−45] ΔG is calculated from the following integral of the isotherms: $\Delta G = -RT\int_0^x (n/x)\,dx$, where n (mol of EtOH or TFE/mol protein) is the amount of sorption as a function of the relative pressure $x = P/P_0$, where P_0 is the saturated vapor pressure. ΔG is expressed in units of kJ/mol. Values of ΔG at different temperatures and vapor pressures can be obtained from the measured isotherm $n(x)$. The enthalpy change is obtained from $\Delta H = -T^2(\partial/\partial T)(\Delta G/T)x$, which is derived from the Gibbs−Helmholtz equation. If $\Delta G(T,x)$ at two different temperatures T_i and T_f is known, then $(\Delta G(T_f, x)/T_f) - (\Delta G(T_i, x)/T_i) = \Delta H(x)((1/T_f) - (1/T_i))$, where $\Delta G(T_i, x)$ and $\Delta G(T_f, x)$ represent the Gibbs free energy change at fixed relative pressure x and at temperatures T_i and T_f, respectively. Here, $\Delta H(x)$ is the enthalpy change at fixed relative pressure x and at an average temperature T, which is given by $1/T = 1/2((1/T_i) + (1/T_f))$. Hence, isotherms measured at 6 and 15 °C, 15 and 25 °C can be employed to estimate the enthalpy change around 10 and 20 °C, respectively. Similarly, the entropy change is calculated from $\Delta S = -((\partial/\partial T)\Delta G)_x$. Again, if $\Delta G(T,x)$ at two different temperatures and T_i and T_f are known from isotherm measurements, we can calculate $\Delta S(T,x)$ as $\Delta S(T,x) = ((\Delta G(T_i, x) - \Delta G(T_f, x))/(T_f - T_i))$. $\Delta S(T,x)$ is the estimated entropy change at an average temperature T given by $1/T = 1/2((1/T_i) + (1/T_f))$. For comparison of ΔG with ΔH and $T\Delta S$ at the same temperature, ΔG at the average temperature T can be obtained by $(\Delta G(T,x)/T) = 1/2((\Delta G(T_i,x)/T_i)) + (\Delta G(T_f,x)/T_f)$.

RESULTS

Sorption Isotherms of Alcohols. Sorption isotherms of EtOH and TFE in dry BSA were measured at 6, 15, and 25 °C. Isotherms of TFE at various levels of BSA hydration were also obtained at 15 and 25 °C.

Figure 1 shows the isotherms of EtOH (Figure 1A) and TFE (Figure 1B) in dry BSA. The insets show isotherms below the relative vapor pressure of $P/P_0 \approx 0.7$ where P is the vapor pressure and P_0 is the saturated vapor pressure of the pure liquid alcohols at the given temperature. Three stages are clearly recognized in the isotherms. Below the first threshold pressure of $P/P_0 \approx 0.15$ for EtOH and $P/P_0 \approx 0.3$ for TFE, isotherms of both alcohols are independent of the temperature and the number of bound alcohol molecules is relatively small, reaching ∼8 EtOH and ∼6 TFE per BSA, in agreement with the number of high-affinity binding sites on serum albumin surface for alcohols and other similar amphiphilic molecules.[46−48] Above this first threshold pressure, isotherms of both alcohols increase rapidly and are greatly enhanced by increasing temperature. The number of bound alcohols far exceeds the number of high-affinity binding sites and increases with alcohol vapor pressure, implying that binding at multiple nonspecific sites takes place.[49−51] In addition, above $P/P_0 \approx 0.7$, a sharp uptake of both alcohols occurs, corresponding to the denaturation of proteins;[52−54] the denaturation is also proven by the irreversibility of desorption isotherms with large hysteresis above $P/P_0 \approx 0.7$ (Supporting Information Figure S1). Hence, the isotherms reveal three steps of alcohol binding. At low vapor pressure, alcohols bind to a few pre-existing high-affinity sites; above a threshold vapor pressure, alcohol binding at multiple nonspecific sites is turned on; at very high vapor pressure, a large number of alcohols bind, causing denaturation of the protein.

Figure 2A shows isotherms of TFE at 15 °C below $P/P_0 = 0.3$ in BSA hydrated at various levels (the complete isotherms can be found in Supporting Information Figure S2A). As the level of protein hydration increases, the nonspecific binding of TFE increases dramatically; the shape of the isotherm gradually changes from sigmoidal ($h = 0.11$) to hyperbolic ($h = 0.32$), indicating a change in alcohol binding pattern, which is discussed later. The threshold level of vapor pressure for nonspecific binding also decreases gradually with increasing hydration level. Figure 2B shows that the threshold level decreases linearly with the hydration level and reaches zero around $h \approx 0.2$. Hydration facilitates TFE to saturate the high-affinity sites and enhances binding at nonspecific sites at lower vapor pressures, showing that hydration promotes alcohol

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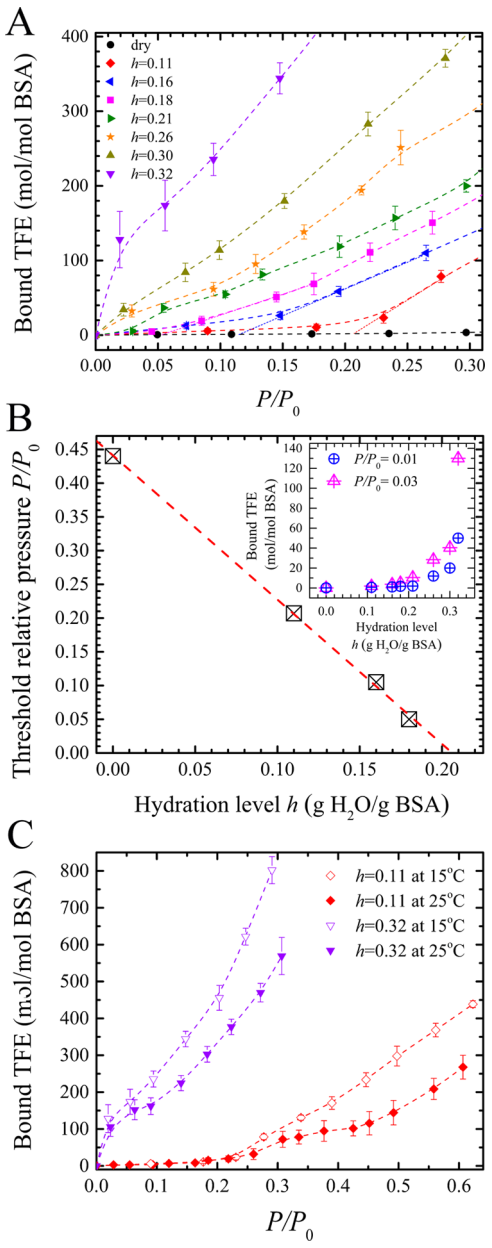

Figure 2. (A) Isotherms of TFE in hydrated BSA at 15 °C below P/P_0 = 0.3 at various hydration levels. Dotted straight lines associated with the isotherms of h = 0.11, 0.16, and 0.18 illustrate how the threshold (the intercept of the dotted line with the horizontal line of y = 0) is determined for a given isotherm associated with nonspecific alcohol binding. (B) The determined alcohol relative vapor pressure threshold is plotted versus h at 15 °C. The threshold decreases linearly with h and reaches zero at $h \approx 0.2$. Inset: The number of bound TFE versus h at 15 °C at P/P_0 = 0.01 and P/P_0 = 0.03. The value of thresholds and the number of bound TFE were determined from (A). (C) Isotherms of TFE in hydrated BSA at h = 0.11, and 15 and 25 °C, and at h = 0.32, and 15 and 25 °C. The isotherms at h = 0.11 show a relative pressure threshold at $P/P_0 \approx 0.25$, while no threshold is seen in isotherms at h = 0.32. At each alcohol vapor pressure, NMR signal was measured five times when the interaction reached equilibrium, and then the standard deviations of NMR peak areas were used to calibrate the error bars in the isotherms.

binding at both types of sites. The inset of Figure 2B further implies that when h > 0.2, nonspecific alcohol binding could take place immediately at very low alcohol vapor pressure and could overwhelm the number of alcohols at high-affinity sites at a high hydration level (h > 0.3). The isotherms of TFE in hydrated BSA at 25 °C show similar behaviors (Supporting Information Figure S2B). Isotherms of TFE in BSA at h = 0.11 and h = 0.32 at 15 and 25 °C are shown in Figure 2C. When h = 0.11, nonspecific alcohol binding only takes place above $P/P_0 \approx 0.25$, while no threshold is observed at h = 0.32. It is interesting to note that nonspecific alcohol binding in hydrated BSA is reduced by increasing temperature, showing a temperature dependence opposite to isotherms in dry BSA.

Sorption Isotherms of Water. Water isotherms in BSA were measured at 6, 15, and 25 °C and are shown in Figure 3.

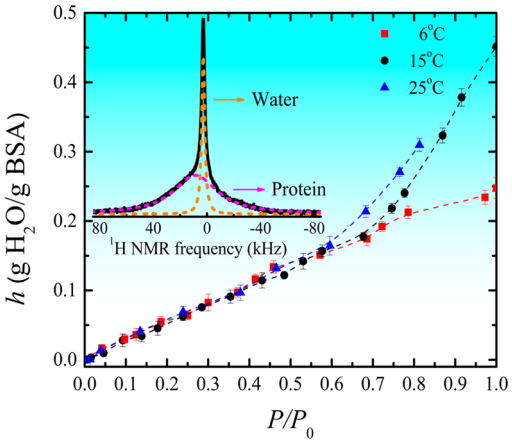

Figure 3. Water sorption isotherms of BSA at 6, 15, and 25 °C. The isotherms show temperature dependence above $h \approx 0.2$. The inset shows a typical ^{1}H NMR spectrum at 25 °C and $h \approx 0.2$. The peak associated with water component and the peak associated with protein component are obtained by Lorentz line fitting. At each water vapor pressure, NMR signal was measured five times when the interaction reached equilibrium, and then the standard deviations of NMR peak areas were used to calibrate the error bars in the isotherms.

Below $h \approx 0.2$ (P/P_0 < ~0.7), isotherms show little temperature dependence, whereas above $h \approx 0.2$, the sorption of water is enhanced by increasing temperature. Similar results were observed in water sorption isotherms on hen egg-white lysozyme,[41] indicating that such behavior could be general in different proteins. It was suggested that below $h \approx 0.2$, water molecules only bind to charged and polar groups on the protein surface.[34,35] Such surface-bound water is able to increase the flexibility of the protein,[34,35,41] also proven by decreasing ^{1}H NMR line width (Supporting Information Figure S3). Interestingly, alcohol sorption isotherms in dry BSA exhibit certain similarities in their temperature dependence (Figure 1).

Gibbs Free Energy, Enthalpy, and Entropy Changes. Changes in Gibbs free energy ΔG, enthalpy ΔH, and entropy $T\Delta S$ associated with alcohol binding at nonspecific sites were calculated (see Methods). Representative results of such binding in dry BSA are summarized in Table 1. Detailed energy diagrams can be found in Supporting Information Figure S4. Inspection of Table 1 reveals that the binding of EtOH and TFE to BSA produces similar thermodynamic changes. First, ΔG is negative, suggesting that the binding is spontaneous. Second, ΔH and $T\Delta S$ are both positive, indicating that above the thresholds, the alcohol binding is completely driven by the favorable entropy change that compensates for the unfavorable enthalpy change. Figure 4 shows the effect of hydration on the thermodynamic changes of

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Table 1. ΔG, ΔH, and $T\Delta S$ of EtOH and TFE Binding to Dry BSA at 10 and 20 °C[a]

	temp (°C)	ΔG (kJ/mol)	ΔH (kJ/mol)	$T\Delta S$ (kJ/mol)
EtOH	10	−2.4 ± 0.4	30.7 ± 3.5	33.1 ± 2.5
	20	−1.7 ± 0.2	26.3 ± 2.1	28.0 ± 2.2
TFE	10	−1.4 ± 0.2	25.9 ± 1.4	27.3 ± 1.3
	20	−1.3 ± 0.4	20.4 ± 2.4	21.7 ± 3.4

[a]Isotherms at 6 and 15 °C, 15 and 25 °C in Supporting Information Figure S1 were used to calculate ΔG, ΔH, and $T\Delta S$ at 10 and 20 °C, respectively (see Methods). Relative vapor pressures in the region where nonspecific binding dominates the isotherms (after completion of binding to high-affinity sites and before denaturation taking place) were used in the calculation (EtOH at 10 °C, $P/P_0 \approx 0.4$; EtOH at 20 °C, $P/P_0 \approx 0.35$; TFE at 10 °C, $P/P_0 \approx 0.55$; TFE at 20 °C, $P/P_0 \approx 0.4$). ΔG, ΔH, and $T\Delta S$ were calculated at such relative vapor pressures and summarized in Table 1. The errors bars of isotherms in Figure 1 were propagated to calculate the uncertainties of ΔG, ΔH, and $T\Delta S$.

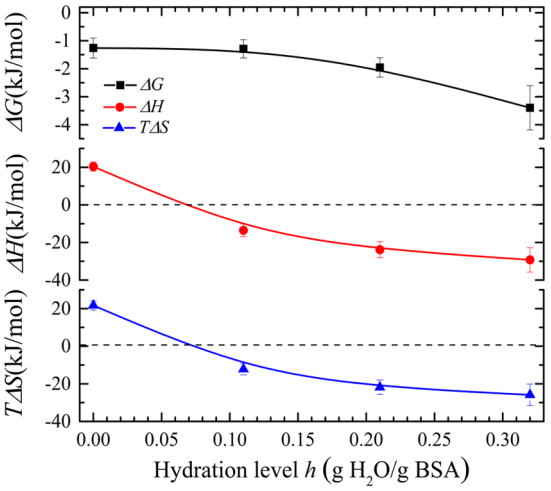

Figure 4. ΔG, ΔH, and $T\Delta S$ of TFE binding in hydrated BSA versus hydration level h at 20 °C. Isotherms at the same hydration levels in Supporting Information Figure S2 were used to calculate ΔG, ΔH, and $T\Delta S$ at 20 °C (see Methods). Relative vapor pressures in the region where nonspecific binding dominates the isotherms (after completion of binding to high-affinity sites and before denaturation taking place) were used in the calculation (dry, $P/P_0 \approx 0.4$; $h = 0.11$, $P/P_0 \approx 0.4$; $h = 0.21$, $P/P_0 \approx 0.2$; $h = 0.32$, $P/P_0 \approx 0.1$). ΔG, ΔH, and $T\Delta S$ were calculated at such relative vapor pressures and plotted. The error bars of isotherms in Supporting Information Figure S2 were propagated to calculate the error bars.

TFE binding. Interestingly, with hydration, both ΔH and $T\Delta S$ became negative, indicating that protein hydration shifts nonspecific alcohol binding from an entropy-driven to an enthalpy-driven process. The increase in $|\Delta G|$ with hydration demonstrates that hydration promotes nonspecific alcohol binding. At high hydration levels ($h > 0.3$), the binding energy ($-\Delta G$) of alcohols at nonspecific regions is slightly smaller than that of alcohols at high-affinity sites, which is around 4.2 kJ/mol.[12,49] Moreover, ΔH and $T\Delta S$ decrease appreciably at low hydration levels, while ΔG decreases appreciably at high hydration levels. It is interesting to note that ΔH and $T\Delta S$ are close to 0 at $h \approx 0.07$, which is around the hydration level at which water fully covers charged groups and starts binding to polar groups on protein surfaces.[34,35] Detailed energy diagrams of TFE binding to BSA at $h = 0.11$, $h = 0.21$, and $h = 0.32$ can be found in Supporting Information Figure S5.

DISCUSSION

High-Affinity and Low-Affinity (Nonspecific) Alcohol Binding. Two types of alcohol binding were revealed by the isotherms in dry and hydrated BSA. One corresponds to the binding of alcohols to a few high-affinity sites and the binding energy is around ∼4.2 kJ/mol;[12,49] the other corresponds to adsorption at multiple low-affinity nonspecific sites and the binding energy is around 1−3.5 kJ/mol (see Table 1 and Figure 4). These two types of binding exhibit different responses to protein hydration and temperature, indicating that they are governed by different binding mechanisms.

Approximately 8 EtOH or 6 TFE bind to the high-affinity sites on dry BSA (Figure 1), and there is no threshold for this type of alcohol binding. Protein hydration can promote the saturation of these high-affinity sites (Figure 2A), while the isotherms show that this type of alcohol binding is independent of temperature (Figures 1 and 2C). These results imply that there are several pre-existing easy-accessible alcohol binding sites on the protein surface.[46−48] These sites are preserved in dehydration process. Alcohol binding to these high-affinity sites is mainly through direct protein−alcohol interactions, such as H-bonds or van der Waals forces, which are minimally affected by the temperature examined here.[55] It appears that these sites are not occupied by water, or water molecules at these sites are easily replaced by alcohols.

In contrast, a large number of alcohols bind to low-affinity nonspecific protein sites above a certain alcohol vapor pressure threshold (Figures 1 and 2A). Protein hydration gradually lowers that threshold, removing it at $h \approx 0.2$, and increases the binding energy, leading to significantly enhanced nonspecific alcohol binding even at very low vapor pressure. Furthermore, this low-affinity nonspecific alcohol binding is very sensitive to temperature (Figures 1 and 2C, and Supporting Information Figure S2). Such strong temperature dependence is attributed to the active involvement of the protein structure in nonspecific binding and is discussed in detail in the following section.

In addition, in fully hydrated proteins, $h > 0.5$ (Figure 3); our results show that the threshold of nonspecific alcohol binding is already removed at such high-level hydration condition and the number of those nonspecifically bound alcohols could dominate (Figure 2B). The binding energy associated with nonspecific alcohol binding at high hydration level also becomes comparable to that of high-affinity alcohol binding (Figure 4). Hence, the present work implies that alcohol binding at multiple low-affinity nonspecific sites could play non-negligible roles in determining alcohols' effects. The method of gradually increasing protein hydration level effectively separates high-affinity and low-affinity (nonspecific) alcohol binding, providing a way to selectively investigate these two types of alcohol binding.

Active Role of Protein in Nonspecific Binding. We have discussed that alcohols can bind to a few high-affinity sites on the protein surface via direct alcohol−protein interactions. Usually, traditional surface binding theories such as Brunauer−Emmett−Teller or Langmuir theory are applied to explain surface adsorption isotherms.[34,35,55] In these theories, the protein is treated as a rigid surface; the corresponding isotherms show little temperature dependence and no thresholds.[55] Therefore, unlike high-affinity alcohol binding, alcohol

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binding at multiple low-affinity nonspecific sites cannot be interpreted by those traditional surface adsorption theories.

There are certain similarities between the temperature dependence of water isotherms (Figure 3) and that of alcohol sorption isotherms in dry proteins (Figure 1). Protein hydration above $h \approx 0.2$ is also strongly enhanced by temperature. Our groups' previous work revealed that those water molecules above $h \approx 0.2$ actually intimately "mix" with the protein structure rather than sitting on the protein surface. Specifically, the elastic constant of the protein is small at high temperature and the increase in elastic energy due to swelling is small; hence, "mixing" of the protein with water molecules is energetically not too costly at high temperature. However, at low temperature, the protein is more rigid with larger elastic constant, and "mixing" with water molecules is more costly in increased elastic energy.[41] The elasticity of the protein is critical to the temperature dependence of water isotherms. Here, our present results suggest that nonspecific alcohol binding, like hydration above $h \approx 0.2$, is essentially a "mixing" process, in which the protein structure is actively involved.

Furthermore, this "mixing" process only occurs above a threshold of alcohol vapor pressure. It is known that dehydration leads to strong protein−protein contacts, hence a rigid structure in the dry protein aggregate.[34,35,56,57] Because of the compact protein aggregate structure, a large number of nonspecific sites may not be available to alcohols. However, alcohols are able to change protein structure,[11−17] and serum albumin has a great conformational adaptability in ligand binding.[38,39,48] Hence, it is likely that the initial alcohol binding at high-affinity sites can disturb intermolecular protein−protein interactions, creating pathways for alcohols to access those nonspecific sites. Subsequently, alcohol molecules start to "mix" with the protein via adsorption at nonspecific sites.

It is seen that this nonspecific alcohol binding to dry protein accompanies positive changes in enthalpy and entropy (Table 1, and Supporting Information Figure S4). Such thermodynamic change is consistent with the active involvement of protein structure in alcohol binding. Nonspecific alcohol binding (and hydration above $h \approx 0.2$), which is essentially a "mixing" process, can largely disrupt protein−protein interactions and rearrange protein conformations,[13,49−51,58] resulting in increased enthalpy of the protein. However, the protein could also gain a large number of degrees of freedom upon this alcohol-induced disruption[56] with increased entropy. Our results show that the entropy gain in the protein surpasses the unfavorable enthalpy change and drives the nonspecific alcohol binding. In addition, it was suggested that in nonaqueous alcohols or high concentration aqueous alcohol solutions, a large number of alcohol molecules can penetrate into the hydrophobic interior of the protein and extensively break the native protein structure, resulting in dramatic conformational changes and even unfolding of the protein.[13,14,19,53,59,60] The denaturation of BSA at higher alcohol pressures (Figure 1 and Supporting Information Figure S1) further proves that those nonspecifically bound alcohols do "mix" with the protein, and if the "mixing" is too extensive, the intramolecular protein interactions are substantially disrupted causing denaturation.

Effects of Hydration on Alcohol Binding. We show that hydration can remove the threshold for nonspecific alcohol binding around $h \approx 0.2$ and significantly change the shape of alcohol adsorption isotherms (Figure 2). In fact, water adsorption in BSA (Figure 3) also changes appreciably around $h \approx 0.2$. This implies that a certain amount of hydration ($h \approx 0.2$) may induce qualitative changes in protein properties, hence affecting alcohol binding. Interestingly, it was also reported that the enzyme activity[34] of proteins is quite different below and above the hydration level around $h \approx 0.2$.

It was suggested that water only binds to high-affinity regions of charged and polar groups on the protein surface below $h \approx 0.2$; above $h \approx 0.2$, water starts to bind loosely to hydrophobic regions of the protein.[34,35] We just discussed that those loosely bound water molecules can actively "mix" with the protein. Significantly, water adsorption at charged and polar protein groups can activate the protein to a more flexible state with reduced elastic constant,[34,35,41] enabling the "mixing" process. This increase in protein elasticity with hydration is further proven by the narrowing of the protein ^{1}H NMR line width in Supporting Information Figure S3. Moreover, infrared measurements and X-ray studies showed that, like alcohol adsorption, water adsorption also accompanies the disruption of protein−protein contacts in solid serum albumin and other proteins.[35,56,61,62] Specifically, the formation of water interaction with charged and polar protein groups is likely to cause pronounced protein configuration rearrangements; when the hydration of those groups is complete, the protein structure is basically identical to that in solution.[56,61] This is consistent with the increase in the protein flexibility with hydration and changes in water isotherms around $h \approx 0.2$ in our work.

The mechanism of water and organic solvents such as alcohols cooperatively changing the protein state was previously suggested.[56,63,64] Therefore, the possible explanation of how hydration promotes alcohol binding is when $h < \sim 0.2$, hydration of charged and polar protein sites only releases part of the protein−protein contacts via disturbing intermolecular protein−protein interactions and alcohols are needed to further "liberate" the protein−protein contacts; hence the threshold for alcohol binding still exists but decreases with hydration level. When $h > \sim 0.2$, the intermolecular protein−protein contacts are largely removed by water; as a consequence, the threshold for alcohol binding disappears. Furthermore, hydration can create new binding space for alcohols,[56] and thus the number of bound alcohols increases dramatically with hydration.

Alcohol sorption isotherms in hydrated BSA show temperature dependence opposite to those in dry BSA (Figures 1B and 2D). In dry proteins, the gain in entropy drives alcohol binding, which should be attributed to the increase in the protein's flexibility with alcohol incorporation. In contrast, with increasing hydration level, the binding process is gradually changed from an entropy-driven to an enthalpy-driven process at a crossover hydration level of $h \approx 0.07$, which is the hydration level where water covers all charged groups and starts binding to polar groups on the protein surface.[34,35] Above that hydration level, it appears that the positive change in the protein's entropy is no longer a dominant factor in driving alcohol binding. With higher hydration, ΔG keeps decreasing and enthalpic stabilization becomes more significant. Both ΔH and $T\Delta S$ decrease appreciably with increased hydration and exhibit strong enthalpy−entropy compensation, which is usually observed in low-affinity binding ligands.[1,9,10,65,66] The decrease of ΔH and $T\Delta S$ is more dramatic at low hydration levels, indicating that such enthalpy-driven alcohol binding is mainly enabled by water molecules bound at charged and polar groups on the protein surface. At high hydration levels, those "mixing" water molecules induce smaller changes in ΔH and

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$T\Delta S$, but effectively reduce the enthalpy−entropy compensation, probably due to the expelling of those water molecules by alcohols. Less enthalpy−entropy compensation causes more negative changes of ΔG at high hydration levels, leading to increased alcohol adsorption.

Difference between Alcohols and Anesthetics. Because of the molecular and functional similarities of alcohols and general anesthetics, it was suggested that they might share similar binding mechanisms.[18,49−51,67] Our groups' recent work elucidated the binding mechanism of halothane and other typical general anesthetics.[40] Halothane cannot directly bind to the protein in the absence of hydration. It only interacts with the protein when $h > 0.3$ and the number of bound halothane is limited to a few binding sites, indicating that halothane only binds to a few specific pockets in the protein and the interaction is enabled by hydration above $h = 0.3$. We suggested that water might be displaced by halothane or form a cap above the binding cavity to assist in the halothane binding. The present study shows that a few alcohols can strongly bind to high-affinity sites by direct interactions even in dry proteins. In addition, protein hydration enables low-affinity nonspecific alcohol binding, and the number of nonspecifically bound alcohols is much larger than that of alcohols bound at high-affinity sites. No such nonspecifically bound halothane molecules were observed. Our data show that the binding mechanisms of alcohols and those of general anesthetics should be examined differently. Nevertheless, water plays a crucial role in the binding of alcohols and general anesthetics such as halothane.

CONCLUSIONS

Great effort has been made to evaluate the effects of structural changes in the water network upon ligand binding. What is little understood is the role of water−protein interaction in ligand binding. Using an NMR-based isotherm measurement approach, we studied the low-affinity binding of alcohols to the globular protein BSA under controlled protein hydration. This technique allowed us to investigate the effect of water−protein interactions on alcohol binding and investigate the specific roles of different hydration water molecules without being obscured by the presence of bulk solvent. The binding thermodynamics of weakly bound alcohols were examined as a function of protein hydration level via the temperature dependence of alcohol adsorption isotherms. We found that alcohol binding is substantially enhanced by hydration. Two types of bound alcohols were clearly identified when the hydration level was below $h \approx 0.2$. One type is alcohol binding at pre-existing high-affinity sites that are limited in numbers (less than 10 per BSA). Although hydration enables alcohol adsorption at these high-affinity sites with lower alcohol vapor pressure, the number remains the same. The second type is alcohol binding at low-affinity nonspecific sites on the protein. In dry protein, this type of alcohol adsorption only occurs above an alcohol vapor pressure threshold. Above the threshold, a large number of alcohol molecules adsorb at nonspecific sites. Water at charged and polar groups on the protein surface was found to be very effective in reducing the threshold pressure and finally removing it at a hydration level of $h \approx 0.2$. Such threshold is probably induced by the strong protein−protein contacts in dry protein aggregates. At the initial stage of hydration, the protein−protein contacts depend strongly on the hydration level until the charged and polar groups are fully hydrated, which corresponds to $h \approx 0.2$. Therefore, the threshold for alcohol adsorption induced by protein−protein contacts is sensitive to the level of hydration. The binding at nonspecific sites was found to be entropy-driven in dry protein but became a fully developed enthalpy-driven process at high hydration levels. These results clearly show that water at charged and polar protein sites ($h \approx 0.2$) plays a crucial role in alcohol binding at nonspecific sites. Although adsorbed water molecules at high hydration levels have smaller effects on alcohol adsorption enthalpy and entropy, they do lead to unmatched enthalpy−entropy compensation, resulting in a more negative Gibbs free energy and enhanced alcohol adsorption. In fact, the number of adsorbed alcohol molecules at low-affinity nonspecific sites far exceeds those at high-affinity sites in fully hydrated proteins. Hence, our work shows the significance of the water−protein interaction in alcohol binding and alcohol's biological actions.

ASSOCIATED CONTENT

Supporting Information

Adsorption−desorption curves of alcohols in dry BSA; adsorption isotherms of trifluoroethanol in BSA with a series of protein hydration levels; changes in protein ^{1}H NMR line width versus hydration level; and changes in Gibbs free energy, enthalpy, and entropy of binding alcohols to dry and hydrated BSA. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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REFERENCES

(1) Breiten, B.; Lockett, M. R.; Sherman, W.; Fujita, S.; Al-Sayah, M.; Lange, H.; Bowers, C. M.; Heroux, A.; Krilov, G.; Whitesides, G. M. Water Networks Contribute to Enthalpy/Entropy Compensation in Protein−Ligand Binding. *J. Am. Chem. Soc.* **2013**, *135*, 15579−15584.

(2) Michel, J.; Tirado-Rives, J.; Jorgensen, W. L. Energetics of Displacing Water Molecules from Protein Binding Sites: Consequences for Ligand Optimization. *J. Am. Chem. Soc.* **2009**, *131*, 15403−15411.

(3) Li, Z.; Lazaridis, T. Water at Biomolecular Binding Interfaces. *Phys. Chem. Chem. Phys.* **2007**, *9*, 573−581.

(4) Levy, Y.; Onuchic, J. N. Water Mediation in Protein Folding and Molecular Recognition. *Annu. Rev. Biophys. Biomol. Struct.* **2006**, *35*, 389−415.

(5) Chaplin, M. Do We Underestimate the Importance of Water in Cell Biology? *Nat. Rev. Mol. Cell Biol.* **2006**, *7*, 861−866.

(6) Barillari, C.; Taylor, J.; Viner, R.; Essex, J. W. Classification of Water Molecules in Protein Binding Sites. *J. Am. Chem. Soc.* **2007**, *129*, 2577−2587.

(7) Shan, Y.; Kim, E. T.; Eastwood, M. P.; Dror, R. O.; Seeliger, M. A.; Shaw, D. E. How Does a Drug Molecule Find Its Target Binding Site? *J. Am. Chem. Soc.* **2011**, *133*, 9181−9183.

(8) Ladbury, J. E. Just Add Water! The Effect of Water on the Specificity of Protein-Ligand Binding Sites and Its Potential Application to Drug Design. *Chem. Biol.* **1996**, *3*, 973−980.

DOI: 10.1021/acs.jpcb.5b00378
J. Phys. Chem. B XXXX, XXX, XXX−XXX

(9) Mobley, D. L.; Dill, K. A. Binding of Small-Molecule Ligands to Proteins: "What You See" Is Not Always "What You Get". *Structure* **2009**, *17*, 489−498.
(10) Baron, R.; McCammon, J. A. Molecular Recognition and Ligand Association. *Annu. Rev. Phys. Chem.* **2013**, *64*, 151−175.
(11) Harris, R. A.; Trudell, J. R.; Mihic, S. J. Ethanol's Molecular Targets. *Sci. Signaling* **2008**, *1*, re7.
(12) Howard, R. J.; Slesinger, P. A.; Davies, D. L.; Das, J.; Trudell, J. R.; Harris, R. A. Alcohol-Binding Sites in Distinct Brain Proteins: The Quest for Atomic Level Resolution. *Alcohol.: Clin. Exp. Res.* **2011**, *35*, 1561−1573.
(13) Dwyer, D.; Bradley, R. Chemical Properties of Alcohols and Their Protein Binding Sites. *Cell. Mol. Life Sci.* **2000**, *57*, 265−275.
(14) Povey, J. F.; Smales, C. M.; Hassard, S. J.; Howard, M. J. Comparison of the Effects of 2, 2, 2-Trifluoroethanol on Peptide and Protein Structure and Function. *J. Struct. Biol.* **2007**, *157*, 329−338.
(15) Shao, Q.; Fan, Y.; Yang, L.; Gao, Y. Q. From Protein Denaturant to Protectant: Comparative Molecular Dynamics Study of Alcohol/Protein Interactions. *J. Chem. Phys.* **2012**, *136*, 115101.
(16) Shao, Q. The Addition of 2,2,2-Trifluoroethanol Prevents the Aggregation of Guanidinium around Protein and Impairs Its Denaturation Ability: A Molecular Dynamics Simulation Study. *Proteins* **2014**, *82*, 944−953.
(17) Trudell, J. R.; Harris, R. A. Are Sobriety and Consciousness Determined by Water in Protein Cavities? *Alcohol.: Clin. Exp. Res.* **2004**.
(18) Sauguet, L.; Howard, R. J.; Malherbe, L.; Lee, U. S.; Corringer, P.-J.; Harris, R. A.; Delarue, M. Structural Basis for Potentiation by Alcohols and Anaesthetics in a Ligand-Gated Ion Channel. *Nat. Commun.* **2013**, *4*, 1697.
(19) Rajan, R.; Balaram, P. A Model for the Interaction of Trifluoroethanol with Peptides and Proteins. *Int. J. Pept. Protein Res.* **1996**, *48*, 328−336.
(20) Chandler, D. Interfaces and the Driving Force of Hydrophobic Assembly. *Nature* **2005**, *437*, 640−647.
(21) Baron, R.; Setny, P.; Andrew McCammon, J. Water in Cavity−Ligand Recognition. *J. Am. Chem. Soc.* **2010**, *132*, 12091−12097.
(22) Hummer, G. Molecular Binding: Under Water's Influence. *Nat. Chem.* **2010**, *2*, 906−907.
(23) Setny, P.; Baron, R.; McCammon, J. How Can Hydrophobic Association Be Enthalpy Driven? *J. Chem. Theory Comput.* **2010**, *6*, 2866.
(24) Ball, P. Water as an Active Constituent in Cell Biology. *Chem. Rev.* **2008**, *108*, 74−108.
(25) Zhong, D.; Pal, S. K.; Zewail, A. H. Biological Water: A Critique. *Chem. Phys. Lett.* **2011**, *503*, 1−11.
(26) Helms, V. Protein Dynamics Tightly Connected to the Dynamics of Surrounding and Internal Water Molecules. *ChemPhysChem* **2007**, *8*, 23−33.
(27) Frauenfelder, H.; Chen, G.; Berendzen, J.; Fenimore, P. W.; Jansson, H.; McMahon, B. H.; Stroe, I. R.; Swenson, J.; Young, R. D. A Unified Model of Protein Dynamics. *Proc. Natl. Acad. Sci. U.S.A.* **2009**, *106*, 5129−5134.
(28) Nucci, N. V.; Pometun, M. S.; Wand, A. J. Mapping the Hydration Dynamics of Ubiquitin. *J. Am. Chem. Soc.* **2011**, *133*, 12326−12329.
(29) Wand, A. J. The Dark Energy of Proteins Comes to Light: Conformational Entropy and Its Role in Protein Function Revealed by Nmr Relaxation. *Curr. Opin. Struc. Biol.* **2013**, *23*, 75−81.
(30) Frederick, K. K.; Marlow, M. S.; Valentine, K. G.; Wand, A. J. Conformational Entropy in Molecular Recognition by Proteins. *Nature* **2007**, *448*, 325−329.
(31) Chia-en, A. C.; Chen, W.; Gilson, M. K. Ligand Configurational Entropy and Protein Binding. *Proc. Natl. Acad. Sci. U.S.A.* **2007**, *104*, 1534−1539.
(32) Marlow, M. S.; Dogan, J.; Frederick, K. K.; Valentine, K. G.; Wand, A. J. The Role of Conformational Entropy in Molecular Recognition by Calmodulin. *Nat. Chem. Biol.* **2010**, *6*, 352−358.
(33) Tzeng, S.-R.; Kalodimos, C. G. Protein Activity Regulation by Conformational Entropy. *Nature* **2012**, *488*, 236−240.
(34) Rupley, J. A.; Careri, G. Protein Hydration and Function. *Adv. Protein Chem.* **1991**, *41*, 37−172.
(35) Kuntz, I., Jr.; Kauzmann, W. Hydration of Proteins and Polypeptides. *Adv. Protein Chem.* **1974**, *28*, 239−345.
(36) Krasowski, M. D.; Harrison, N. L. The Actions of Ether, Alcohol and Alkane General Anaesthetics on Gabaa and Glycine Receptors and the Effects of Tm2 and Tm3Mutations. *Br. J. Pharmacol.* **2000**, *129*, 731−743.
(37) Kaminsky, L. S.; Fraser, J. M.; Seaman, M.; Dunbar, D. Rat Liver Metabolism and Toxicity of 2, 2, 2-Trifluoroethanol. *Biochem. Pharmacol.* **1992**, *44*, 1829−1837.
(38) Majorek, K. A.; Porebski, P. J.; Dayal, A.; Zimmerman, M. D.; Jablonska, K.; Stewart, A. J.; Chruszcz, M.; Minor, W. Structural and Immunologic Characterization of Bovine, Horse, and Rabbit Serum Albumins. *Mol. Immunol.* **2012**, *52*, 174−182.
(39) Peters, T., Jr. *All About Albumin: Biochemistry, Genetics, and Medical Applications*; Academic Press: New York, 1995.
(40) Wang, H.-J.; Kleinhammes, A.; Tang, P.; Xu, Y.; Wu, Y. Critical Role of Water in the Binding of Volatile Anesthetics to Proteins. *J. Phys. Chem. B* **2013**, *117*, 12007−12012.
(41) Wang, H.-J.; Kleinhammes, A.; Tang, P.; Xu, Y.; Wu, Y. Temperature Dependence of Lysozyme Hydration and the Role of Elastic Energy. *Phys. Rev. E* **2011**, *83*, 031924.
(42) Myers, A. Thermodynamics of Adsorption in Porous Materials. *AIChE J.* **2002**, *48*, 145−160.
(43) Hill, T. L. Statistical Mechanics of Adsorption. V. Thermodynamics and Heat of Adsorption. *J. Chem. Phys.* **1949**, *17*, 520.
(44) Hill, T. L. Statistical Mechanics of Adsorption. Ix. Adsorption Thermodynamics and Solution Thermodynamics. *J. Chem. Phys.* **1950**, *18*, 246.
(45) Keren, R.; Shainberg, I. Water Vapor Isotherms and Heat of Immersion of Na-and Ca-Montmorillonite Systems. III. Thermodynamics. *Clays Clay Miner.* **1980**, *28*, 204−210.
(46) Reynolds, J. A.; Herbert, S.; Steinhardt, J. Binding of Some Long-Chain Fatty Acid Anions and Alcohols by Bovine Serum Albumin. *Biochemistry* **1968**, *7*, 1357−1361.
(47) Polet, H.; Steinhardt, J. Binding-Induced Alterations in Ultraviolet Absorption of Native Serum Albumin. *Biochemistry* **1968**, *7*, 1348−1356.
(48) Ghuman, J.; Zunszain, P. A.; Petitpas, I.; Bhattacharya, A. A.; Otagiri, M.; Curry, S. Structural Basis of the Drug-Binding Specificity of Human Serum Albumin. *J. Mol. Biol.* **2005**, *353*, 38−52.
(49) Eckenhoff, R. G.; Johansson, J. S. Molecular Interactions between Inhaled Anesthetics and Proteins. *Pharmacol. Rev.* **1997**, *49*, 343−368.
(50) Urban, B. W.; Bleckwenn, M.; Barann, M. Interactions of Anesthetics with Their Targets: Non-Specific, Specific or Both? *Pharmacol. Ther.* **2006**, *111*, 729−770.
(51) Eckenhoff, R. G. Do Specific or Nonspecific Interactions with Proteins Underlie Inhalational Anesthetic Action? *Mol. Pharmacol.* **1998**, *54*, 610−615.
(52) Liu, R.; Qin, P.; Wang, L.; Zhao, X.; Liu, Y.; Hao, X. Toxic Effects of Ethanol on Bovine Serum Albumin. *J. Biochem. Mol. Toxicol.* **2010**, *24*, 66−71.
(53) Yoshikawa, H.; Hirano, A.; Arakawa, T.; Shiraki, K. Effects of Alcohol on the Solubility and Structure of Native and Disulfide-Modified Bovine Serum Albumin. *Int. J. Biol. Macromol.* **2012**, *50*, 1286−1291.
(54) Hirota, N.; Goto, Y.; Mizuno, K. Cooperative A-Helix Formation of B-Lactoglobulin and Melittin Induced by Hexafluoroisopropanol. *Protein Sci.* **1997**, *6*, 416−421.
(55) de Boer, J. H. *The Dynamical Character of Adsorption*, 2nd ed.; Clarendon Press: Oxford, UK, 1968.
(56) Permîâkov, E. A. e.; Uversky, V. N. *Methods in Protein Structure and Stability Analysis: Vibrational Spectroscopy*; Nova Publishers: New York, 2007.

DOI: 10.1021/acs.jpcb.5b00378
J. Phys. Chem. B XXXX, XXX, XXX−XXX

(57) Fernández, A.; Scheraga, H. A. Insufficiently Dehydrated Hydrogen Bonds as Determinants of Protein Interactions. *Proc. Natl. Acad. Sci. U.S.A.* **2003**, *100*, 113−118.
(58) Sirotkin, V. A.; Komissarov, I. A.; Khadiullina, A. V. Hydration of Proteins: Excess Partial Volumes of Water and Proteins. *J. Phys. Chem. B* **2012**, *116*, 4098−4105.
(59) Haris, P. I.; Severcan, F. FTIR Spectroscopic Characterization of Protein Structure in Aqueous and Non-Aqueous Media. *J. Mol. Catal. B: Enzym.* **1999**, *7*, 207−221.
(60) Carrotta, R.; Manno, M.; Giordano, F. M.; Longo, A.; Portale, G.; Martorana, V.; San Biagio, P. L. Protein Stability Modulated by a Conformational Effector: Effects of Trifluoroethanol on Bovine Serum Albumin. *Phys. Chem. Chem. Phys.* **2009**, *11*, 4007−4018.
(61) Grdadolnik, J.; Marechal, Y. Bovine Serum Albumin Observed by Infrared Spectrometry. Ii. Hydration Mechanisms and Interaction Configurations of Embedded H2o Molecules. *Biopolymers* **2001**, *62*, 54−67.
(62) Takayama, Y.; Nakasako, M. A Few Low-Frequency Normal Modes Predominantly Contribute to Conformational Responses of Hen Egg White Lysozyme in the Tetragonal Crystal to Variations of Molecular Packing Controlled by Environmental Humidity. *Biophys. Chem.* **2011**, *159*, 237−246.
(63) Klemm, W. Biological Water and Its Role in the Effects of Alcohol 1. *Alcohol (N. Y., NY, U. S.)* **1998**, *15*, 249−267.
(64) Sirotkin, V. A.; Faizullin, D. A. Interaction Enthalpies of Solid Human Serum Albumin with Water−Dioxane Mixtures: Comparison with Water and Organic Solvent Vapor Sorption. *Thermochim. Acta* **2004**, *415*, 127−133.
(65) Bissantz, C.; Kuhn, B.; Stahl, M. A Medicinal Chemist's Guide to Molecular Interactions. *J. Med. Chem.* **2010**, *53*, 5061−5084.
(66) Williams, D. H.; Stephens, E.; O'Brien, D. P.; Zhou, M. Understanding Noncovalent Interactions: Ligand Binding Energy and Catalytic Efficiency from Ligand-Induced Reductions in Motion within Receptors and Enzymes. *Angew. Chem., Int. Ed.* **2004**, *43*, 6596−6616.
(67) Bhattacharji, A.; Klett, N.; Go, R. C. V.; Covarrubias, M. Inhalational Anaesthetics and N-Alcohols Share a Site of Action in the Neuronal Shaw2 Kv Channel. *Br. J. Pharmacol.* **2010**, *159*, 1475−1485.

DOI: 10.1021/acs.jpcb.5b00378
J. Phys. Chem. B XXXX, XXX, XXX−XXX