Steric Hindrance Is Not Required for n-Alkanol Cutoff in Soluble Proteins

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ABSTRACT

A loss of potency as one ascends a homologous series of compounds (cutoff effect) is often used to map the dimensions of binding sites on a protein target. The implicit assumption of steric hindrance is rarely confirmed with direct binding measurements, yet other mechanisms for cutoff exist. We studied the binding and effect of a series of n-alkanols up to hexadecanol (C16) on two model proteins, BSA and myoglobin (MGB), using hydrogen-tritium exchange and light scattering. BSA binds the n-alkanols specifically and, at 1 mM total concentration, is stabilized with increasing potency up to decanol (C10), where a loss in stabilizing potency occurs. Cutoff in stabilizing potency is concentration-dependent and occurs at progressively longer n-alkanols at progressively lower total n-alkanol concentrations. Light scattering measurements of n-alkanol/BSA solutions show a smooth decline in binding stoichiometry with increasing chain length until C14–16, where it levels off at ~2:1 (alkanol:BSA). MGB does not bind the n-alkanols specifically and is destabilized by them with increasing potency until C10, where a loss in destabilizing potency occurs. Like BSA, MGB demonstrates a concentration-dependent cutoff point for the n-alkanols. Derivation of the number of methylenes bound at K_D and the free energy contribution per bound methylene showed that no discontinuity existed to explain cutoff, rendering steric hindrance unlikely. The data also allow an energetic explanation for the variance of the cutoff point in various reductionist systems. Finally, these results render cutoff an untenable approach for mapping binding site stericities in the absence of complementary binding measurements, and a poor discriminator of target relevance to general anesthesia.

Included in the unexplained phenomenology surrounding anesthetic action is the cutoff effect. This term refers to an increase in anesthetic potency as one ascends a homologous series of either n-alkanes or alkanols (or others) to a point where a loss of potency, or at least no further increase, occurs (Ferguson, 1939). For the n-alkanes, cutoff occurs at about decane (C10), whereas in the n-alkanol series, it occurs at about dodecanol (C12; Miller and Alifimoff, 1998). Initially, the cutoff effect was thought to reflect a decrease in the lipid solubility of the longer chain hydrocarbons, such that the Overton Meyer rule (relating solubility in olive oil to anesthetic potency) would not be violated. Subsequently, hydrocarbons longer than cutoff have been found to solubilize exceedingly well into lipid membranes, perhaps rendering such a site an unlikely candidate for mediating important events underlying anesthetic action (Franks and Lieb, 1986; Miller et al., 1989), although partitioning per se may not correlate with perturbations in the bilayer. More recently, proteins have received renewed attention as direct targets of anesthetics (Franks and Lieb, 1994; Eckenhoff and Johansson, 1997). Accordingly, ethanol has been found to bind saturably to biological membranes (Channareddy et al., 1996), and alcohol cutoff has been identified in lipid-free protein systems (Franks and Lieb, 1985). This suggests that direct binding of alcohols to some relevant protein may underlie their actions. If this is true, the simplest interpretation of cutoff is that the binding site on the protein is limited in size and cannot accommodate the larger alcohols, the binding constants of which should, therefore, sharply decrease. Such a steric hindrance mechanism is assumed when investigators use the cutoff effect to map the size of alcohol binding sites and the effects of site-directed mutations on puted clefts or cavities in membrane protein (Franks and Lieb, 1985; Alifimoff et al., 1989; Curry et al., 1991; Peoples and Weight, 1995; Wick et al., 1998). One problem, however, is that binding of these long-chain alcohols has been difficult to measure, and there are no examples where investigators have confirmed the assumption of steric hindrance with direct binding measurements. It also is relevant to note that the aqueous solubility of long-chain alkanes and alkanols decreases dramatically as one ascends the homologous series, in accor-

ABBREVIATIONS: MGB, myoglobin; GdnCl, guanidine HCl; ΔG, Gibb’s free energy; ΔΔG, change in Gibbs free energy; PFr, protection factor ratio.
dance with the unfavorable hydration of methylene. Thus, unless the free concentration of an alcohol can reach a level where significant occupancy of binding sites occurs, a decrease in effect (cutoff) that is indistinguishable from steric hindrance could occur without any decrease in binding constants (Raines and Miller, 1994). Aside from this aqueous solubility-dominated mechanism for cutoff, more subtle mechanisms may exist that also do not invoke steric hindrance. For example, the progressively more hydrophobic alkanes or alkanols may begin to occupy progressively less specific protein sites, which might be expected to oppose actions resulting from occupancy of more specific sites (Eckenhoff and Tanner, 1998). We studied the phenomenon of cutoff for n-alkanols in two soluble proteins, BSA and myoglobin (MGB) using hydrogen exchange as a measure of specific binding and light scattering as a measure of binding stoichiometry. Monitoring the exchange of a protein’s slowest, or most protected, hydrogens provides a measure of protein stability (Bai et al., 1994). While not a direct measurement of binding, stabilization of a protein is nonetheless an unambiguous measure of a preferential interaction between a ligand and features formed by native folded structure, and is independent of protein activity. Furthermore, it does not rely on a particular reporter group on the ligand or protein to indicate an interaction.

**Experimental Procedures**

**Materials.** BSA (99%, essentially fatty acid-free) and horse MGB were purchased from Sigma Chemical Co. (St. Louis, MO) and used without further purification. The series of n-alkanols from propanol (C3) to hexadecanol (C16) also were purchased from Sigma, and 0.1-M stock solutions in methanol were used to introduce the alcohols into the protein solutions. Tritiated water (specific activity = 100 mCi/ml) was purchased from Amersham (Arlington Heights, IL). All other chemicals were of reagent grade.

**Hydrogen Exchange.** Proteins were dissolved at −20 mg/ml in 0.1 M sodium phosphate buffer at pH 8 from 9 with 1 M guanidine HCl (GdnCl), and incubated with about 5 mCi [3H]OH for at least 18 h at room temperature. The high pH accelerates amide hydrogen exchange (Englander and Englander, 1994) in exposed amide groups, and 1 M GdnCl reversibly shifts the folded/unfolded equilibrium of these proteins so that internal amide hydrogens are exposed to solvent more often. This ensures complete equilibration of all exchangeable amide hydrogens with solvent hydrogens. Liquid scintillation counting of small aliquots allowed calculation of the specific activity of [3H] for the determination of the number of hydrogens/protein molecule. Exchange-out was initiated by removing free [3H]OH from the protein solution with a PD-10 (Sigma) gel filtration column, which also facilitated the switch to exchange-out buffer. The pH and GdnCl concentration of this buffer was adjusted to focus on the slow hydrogens (the last 5–10% to exchange-out) in a convenient time window (less than 7 h). Thus, exchange-out conditions were 0.1 M sodium phosphate at pH 7.4 with 0.5 M GdnCl at 37°C. After recovery from the PD-10 column, the protein solutions were immediately transferred to tubes containing the exchange-out buffer with given concentrations of n-alkanol, vortexed, and incubated at 37°C. Aliquots of this solution (average protein concentration, −20 μM) were delivered into 2°C 10% trichloroacetic acid at timed intervals. The precipitated protein was immediately separated by vacuum filtration (Whatman GF/B filters) and washed with 8 ml of 1% TCA at 2°C. The entire precipitation/filtration procedure was routinely accomplished in 10 s or less, and further exchange of hydrogens in this brief time period was excluded as a possibility by comparing retained activity in some parallel protein samples run through a second column according to the technique of Englander and Englander (1994). In some experiments with the longest, least soluble alkanols, the [3H]-loaded protein was first incubated with the alkanol prior to initiation of exchange out, so that on dilution in exchange-out buffer, binding to the protein (−20 μM) would not deplete the free alkanol concentration and slow the re-equilibration. After soaking the filters with fluor overnight, retained [3H] (bound to the protein) was determined by scintillation counting. Protein concentration was determined from the absorbance at 280 nm using extinction coefficients of 45,000 cm−1M−1 for BSA and 30,400 cm−1M−1 for MGB.

**Binding Stoichiometry.** The limited water solubility of long-chain alkanols dictates that micelles will initially form when the solubility limit is surpassed. The formation of micelles can be monitored by light scattering. Thus, by subtracting the total alkanol concentration required to initiate light scattering in buffer solutions without protein from that in solutions with protein, an estimate of bound alkanol is derived, permitting calculation of the binding stoichiometry at a saturated alkanol concentration. Methanolic 0.1- or 0.5-M solutions of C8 through C14 were used to introduce equivalents of n-alkanol to 0.1 mM BSA, MGB, or control (no protein) solutions in 0.1 mM K2PO4, pH 7.2. The solutions were allowed to equilibrate, with occasional mixing, for 1 h, then examined with a Beckman DU-65 spectrophotometer at 400 nm (475 nm for MGB because of heme absorption at 400 nm) for light scattering. Binding stoichiometry at maximal n-alkanol solubility was determined from the number of alkanol equivalents added before a concentration-dependent increase in scattering was observed.

**Data Analysis.** Protection factors for given hydrogens were determined from the exchange-out curves. Assuming horizontal equivalence of hydrogen exchange (the nth hydrogen to exchange is the same hydrogen with and without alcohol, or at least part of the same unfolding unit), protection factor ratios were estimated by dividing the time required for a given hydrogen to exchange under the two conditions (e.g., with and without alcohol), and were determined for the last three to five hydrogens in common for the two conditions. Protection factor ratios (PFr) were then averaged and ΔΔG determined using ΔΔG = −RTln(PFr), where R is the gas constant (1.98 cal/mol/deg K) and T is the temperature in Kelvins. ΔΔG represents the change in free energy of the folded versus the unfolded state in the presence of the test compound as compared to the control condition. Negative ΔΔG values reflect protein stabilization (slower exchange) and positive values indicate destabilization (faster exchange).

**Results**

At least three different total concentrations of each n-alkanol were examined for each of the two proteins. All of the n-alkanols stabilized BSA at all concentrations, and there was a progressive increase in potency for stabilization of BSA with longer chain alcohols, until the added concentration exceeded the maximum solubility for that n-alkanol (Fig. 1). The cutoff point at 1 mM total n-alkanol concentration was decanol (C10), at 0.3 mM it was dodecanol (C12), and at 0.1 mM it was tetradecanol (C14). Also evident from these figures is that the maximum protein stabilization decreased with increasing chain length, so that if an arbitrary free energy input of about 1 kcal/mol is necessary to produce a given change in protein activity, then Fig. 2 shows that there is a progressive increase in n-alkanol potency for achieving this ΔG, until about C12. No concentration of C13 could produce a 1.0 kcal/mol stabilization of BSA. If the free energy input required for alteration of protein activity is larger than this, the cutoff will occur with shorter alkanols, but if a smaller free energy change is required, then the cutoff point would occur at longer chain lengths (e.g., C14 for a ΔΔG of 0.5 kcal/mol)
kcal/mol). Figure 1 also shows that the cutoff behavior is analogous for MGB, except that the direction of the free energy change was opposite (positive $\Delta \Delta G$; destabilization).

Molar equivalents of $n$-alkanols (using concentrated methanolic solutions of $n$-alkanols longer than octanol) were added to either buffer or to BSA solutions, and the samples were then monitored for an increase in light scattering at 400 nm attributable to the formation of alkanol micelles as the maximum water solubility was exceeded. Figure 3 shows clear evidence of binding to BSA, but not to MGB for a typical plot with C10. Plotting the BSA data for all $n$-alkanols longer than C8, Fig. 4 shows a progressive fall in the stoichiometry at saturated $n$-alkanol concentrations. Thus, while the binding stoichiometry was 13:1 (alkanol:BSA) at C8 (octanol), it was 3:1 at C14 (tetradecanol) and 2.5:1 at C16 (hexadecanol). Combined with the maximum $\Delta \Delta G$ at the saturated $n$-alkanol concentration, these stoichiometric data allowed calculation of the $\Delta \Delta G$ per methylene, which remained relatively constant at 17 to 20 cal/mol of protein per methylene, essentially independent of alkanol chain length (Fig. 5).

To calibrate the importance of $n$-alkanol water solubility on protein stabilization, the number of methylenes bound to BSA at $K_D$ was derived from the stoichiometry measurements at maximum solubility and the extrapolated binding constants from the literature (Ray et al., 1966). If steric hindrance precludes binding of the longer chain alkanols, a sharp decrease in the number of methylenes bound at the calculated $K_D$ for some alkanol length would be predicted. We used the Hill equation for these calculations, but because the degree of cooperativity in alcohol binding is uncertain, Hill coefficients from 0.5 to 2 were used and the results shown in Fig. 6. This figure shows a small continuous decrease in the number of methylenes bound at $K_D$ across the entire C8-C14 range for Hill numbers of 1 and 2, but essentially no change for a Hill number of 0.5. Between C14 and C16, however, the data predict an increase in the number of methylenes bound, especially for higher degrees of cooperativity, which should have resulted in a larger $\Delta \Delta G$ for the C16/BSA interaction. That this was not observed is most easily explained by the

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**Fig. 1.** Effect of different total concentrations of $n$-alkanols on stability of BSA or MGB, as reflected by the $\Delta \Delta G$. Positive values reflect destabilization (MGB; closed symbols), and negative values indicate stabilization (BSA; open symbols). Diamonds represent a total $n$-alkanol concentration of 10 mM; squares, 1 mM; triangles, 0.3 mM; and circles, 0.1 mM.

**Fig. 2.** The total concentration of $n$-alkanol required to stabilize $\sim20 \mu$M BSA by 1 kcal/mol versus the $n$-alkanol chain length.

**Fig. 3.** Stoichiometry of decanol/BSA or decanol/MGB binding using light scattering at 400 nm for BSA (■) and 475 nm for MGB (○). The open symbols represent light scattering due to decanol added to buffer without protein.

**Fig. 4.** Stoichiometry of $n$-alkanol binding to BSA at saturated $n$-alkanol concentration as a function of $n$-alkanol chain length.
fact that the maximum water solubility of C16 is less than the $K_D$.

**Discussion**

These data show that there is an increasing potency of the $n$-alkanol series for stabilizing BSA, but that the low aqueous solubility of the members longer than C10 limit the occupancy of $n$-alkanol binding sites, despite progressively increasing binding constants. For a soluble protein that does not significantly bind the alcohols studied, i.e., myoglobin, a progressive increase in potency for destabilization was observed until, again, a water solubility-limited cutoff effect occurred at about C10. Taken together, these results strongly suggest that solubility issues dominate the $n$-alkanol cutoff effect. This is consistent with the observed shift in cutoff point in other homologous series. For example, relative to the C13–14 in vivo cutoff point for the saturated $n$-alkanols, the less soluble $n$-alkane series have a shorter cutoff point (C10; Miller and Alifimoff, 1998), and the more soluble unsaturated $n$-alkanol series have a longer cutoff point (C15; Pringle et al., 1981). Also, similar in vivo studies with the cycloalkanemethanol series showed that molecular volume was better related to cutoff than length (Raines et al., 1993), again suggesting that solubility, rather than steric, controlled cutoff. Finally, similar conclusions were reached with the alkane series in in vivo potency experiments (Liu et al., 1993). Thus, steric hindrance as a mechanism for cutoff cannot be assumed, as it has in several recent studies on the mechanism of alcohol action (Franks and Lieb, 1985; Alifimoff et al., 1989; Curry et al., 1991; Peoples and Weight, 1995; Wick et al., 1998). At least, independent binding measurements are required to draw valid inferences about the steric features of proposed binding sites.

Our results also show that cutoff in stability is concentration-dependent (Fig. 1) and that the ultimate effect of alcohols on protein activity will depend on the energetics of the conformational equilibrium between resting and active states. Should a large change in free energy be required to significantly populate the active protein conformer, then our results suggest that cutoff will occur at a shorter alkanol length, where much higher degrees of occupancy (of either specific or nonspecific sites) can be achieved because of higher solubility. This is consistent with Ferguson’s observation that anesthetic-resistant organisms tend to exhibit cutoff at shorter chain lengths in a homologous series than anesthetic-sensitive organisms (Ferguson, 1939). Conversely, very small energetic differences between resting and active states would predict that cutoff should occur at longer $n$-alkanol chain lengths. Differences in cutoff ranging from C4 to C16 have been observed. It is interesting to note that in the case of firefly luciferase, a relatively unstable soluble protein ($T_m \sim 45^\circ C$), cutoff for inhibition of activity occurs at C14–16 (Franks and Lieb, 1985; Ueda and Suzuki, 1998). The inhibitory ligand-gated ion channels have a somewhat lower cutoff point, C12 to C13, whereas the excitatory ligand-gated ion channels and the 7-helix G-protein coupled receptors are lower, C4 to C9 (Korpi et al., 1998). However, the situation could be very different for membrane proteins because their conformational equilibrium is highly dependent on the character of the surrounding lipid, and the alkanols partition extensively into lipid bilayers. Despite partitioning, the effect of long chain $n$-alkanols on membrane order parameter (Miller et al., 1989) and free volume (Mitchell et al., 1996) shows discontinuity between C8 and C12. Thus, it remains possible that lipid-mediated effects of long chain alkanols dominate in the case of membrane proteins.

The stabilization of BSA clearly represents specific binding to the native folded form of BSA and is consistent with known binding behavior of this protein (Ray et al., 1966). Destabilization of MGB is interpreted to represent preferential binding to less folded or completely unfolded forms of this protein (Eckenhoff and Tanner, 1998). Because the population of these less folded forms is expected to be very low under the conditions of our experiments, it was predicted that there should be no evidence of alkanol binding to MGB in the light-scattering experiments. That hydrogen exchange can detect changes in such a small population of protein molecules relates to the fact that exchange of slow hydrogens can occur only from the less folded conformers. Thus, the observation that significant destabilization can occur in the appar-
ent absence of binding is reconcilable when one considers that the methods focus on different populations of protein conformers. Solubility-dominated cutoff was observed for both specific and nonspecific interactions (stabilization of BSA versus destabilization of MGB). Because we have recently shown that specific interactions model anesthetic phenomenology better than nonspecific interactions (Eckenhoff, 1998), the cutoff effect alone seems to have limited utility as a criterion for establishing the physiological relevance of individual protein targets.

The above interpretations rest, in part, on the assumption that n-alkanol/BSA binding constants continue to increase past C12, where they have been measured with equilibrium dialysis techniques. This assumption is reasonable, however, based on the binding stoichiometry determinations. Hexadecanol (C16), despite having a maximal aqueous solubility of 0.5 μM, 6-fold lower than that of C14, showed approximately the same binding stoichiometry. This is most readily explained by an increased binding affinity of the longer alkanol. Also, it is well known that progressively higher affinity binding sites for fatty acids up to C18 exist in the serum albumins, giving even further confidence that the binding constants for the long-chain alcohols increase similarly.

Another mechanism that could contribute to cutoff is suggested by the data. Note from Fig. 1 that total concentrations of the longer chain alkanols that exceed maximum water solubility stabilized BSA less than lower concentrations of the same n-alkanols. One interpretation is that as the concentration of these more hydrophobic ligands increases, additional but nonspecific binding sites on unfolded protein conformers are recruited, contributing to destabilization and therefore antagonizing the stabilizing interactions produced through specific interactions at other sites. Again, depending on the free energy change required to influence activity, antagonism of stabilizing interactions through recruitment of additional destabilizing interactions (sites) would be expected to contribute to cutoff. The MGB results confirm that destabilization can occur in soluble protein at low n-alkanol concentration. Thus, the overall effect of alkanols on a protein with specific binding sites will be a balance of simultaneous stabilizing and destabilizing influences, and will depend on the number and affinity of specific sites, native protein stability, and protein size and hydrophobicity. A similar mechanism has been invoked to explain the loss of stabilizing potency of progressively longer chain fatty acids on serum albumin (Andrew Shrake, Food and Drug Administration, Bethesda, MD) (personal communication). It should also be noted that such a mechanism could explain cutoff effects at alkanol concentrations less than their maximum solubility in water, and reproduce efficacy-limited cutoff (as distinguished from solubility-limited cutoff; Raines and Miller, 1994). Likewise, it could explain the absence of anesthetic effect in the recently described group of nonimmobilizers (Koblin et al., 1994; Eckenhoff, 1998).

Finally, the conclusions reached here are not limited to the specific protein models used, in that the effect of a ligand on stability of soluble proteins simplifies to the binding interaction, and not the more complex relationship between binding and activity. Thus, despite the fact that neither BSA nor MGB can be a functionally relevant target of the inhaled anesthetics, the interactions studied here represent more proximal steps in the overall interaction and therefore should apply equally well to other soluble proteins.

In summary, steric hindrance as a structural mechanism for n-alkanol potency cutoff cannot be assumed without direct binding measurements. Solubility considerations dominate the cutoff effect, allowing it to become apparent in both specific and nonspecific interactions and thus limiting its utility in exploring anesthetic mechanisms of action.

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References

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