Differential Halothane Binding and Effects on Serum Albumin and Myoglobin

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ABSTRACT To understand further the weak molecular interactions between inhaled anesthetics and proteins, we studied the character and dynamic consequences of halothane binding to bovine serum albumin (BSA) and myoglobin using photoaffinity labeling and hydrogen-tritium exchange (HX). We find that halothane binds saturably and with submillimolar affinity to BSA, but either nonspecifically or with considerably lower affinity to myoglobin. Titration of halothane binding with guanidine hydrochloride suggested more protection of binding sites from solvent in BSA as compared with myoglobin. Protection factors for slowly exchanging albumin hydrogens are increased in a concentration-dependent manner by up to 27-fold with 10 mM halothane, whereas more rapidly exchanging groups of albumin hydrogens have either unaltered or decreased protection factors. Protection factors for slowly exchanging hydrogens in myoglobin are decreased by halothane, suggesting destabilization through binding to an intermediate or completely unfolded conformer. These results demonstrate the conformation dependence of halothane binding and clear dynamic consequences that correlate with the character of binding in these model proteins. Preferential binding and stabilization of different conformational states may underlie anesthetic-induced protein dysfunction, as well as provide an explanation for heterogeneity of action.

INTRODUCTION

It is now clear that volatile anesthetics can influence a wide variety of biological functions, both at the integrated and at the reductionist level (Alifimoff and Miller, 1993). However, it is less clear, and in fact an issue of considerable debate, whether these widespread effects are mediated through direct interactions with the protein component of such systems or through actions on other components such as lipid. Until recently, there have been few unambiguous data for inhalational anesthetic binding to protein, a prerequisite for a direct effect. A host of functional studies using lipid-free preparations of firefly luciferase strongly suggest a direct effect (Franks and Lieb, 1984), and halothane photolabeling suggested specific binding (Evers et al., 1995). On the other hand, a recent calorimetry study (Chiou and Ueda, 1994) showed that anesthetics destabilize this enzyme, suggesting the mechanism for inhibition of activity is preferential binding to an unfolded conformer rather than specific binding to the native, folded state. In other soluble proteins, such as serum albumin, ^{19}F-NMR (Dubois et al., 1993), gas chromatographic partitioning analysis (Dubois and Evers, 1992), and photoaffinity labeling (Eckenhoff and Shuman, 1993) have all demonstrated saturable binding of volatile anesthetics that disappears on unfolding the protein with low pH conditions. Although the location of the specific halothane binding sites on bovine serum albumin (BSA) was recently suggested by tryptophan fluorescence quenching (Johansson et al., 1995), and then confirmed with direct photoaffinity labeling (Eckenhoff, 1996), it is still not clear whether these sites are a feature of the native, folded state or whether they predominantly exist in a less folded, molten state. Although differential scanning calorimetry (DSC) experiments suggest stabilization of BSA by volatile anesthetics (Tanner et al., 1997), thermal unfolding of this protein is not reversible, making the interpretation of such results difficult. Whether anesthetics stabilize or destabilize proteins is of fundamental importance to an understanding of their mechanism of action; differential binding to different conformational states surely underlies anesthetic action and perhaps the subtly unique actions of different anesthetics.

In this study, we examine the relationship between binding and protein stability in two soluble carrier proteins, serum albumin and myoglobin. We use hydrogen exchange (HX) to measure protein stability/dynamics for several reasons. First, because exchange of the slowest hydrogens occurs on exposure of these amide groups to solvent, their exchange kinetics should reflect more complete unfolding events and may be used as a measure of folded stability (Bai et al., 1994). Furthermore, changes in HX rate are observed with small shifts in the folded/unfolded equilibrium that would produce no detectable change in the heat capacity or optical signals (such as circular dichroism spectroscopy). Second, HX can be performed under isothermal conditions, avoiding the issue of unfolding reversibility and the temperature dependence of binding enthalpy. Finally, concentrations of volatile anesthetics are easier to control in the typical HX experiment than in DSC, again because of the isothermal conditions. Our experiments continue to use photoaffinity labeling (Eckenhoff and Shuman, 1993) to measure binding, as it is the only available technique for separation of specific from nonspecific binding for the inhaled anesthetics, requires far less protein than ^{19}F-NMR

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or gas chromatographic partitioning, and does not require proximity of the anesthetic binding site to a reporter group, such as an aromatic residue or spin label. Although photolabeling is commonly used for identification of proteins that bind the ligand preferentially, or for localization of binding sites within a protein, it can also be used to estimate binding parameters (K_D, B_{max}, and Hill number) by titrating label incorporation against unlabeled ligand concentration (competition assay).

MATERIALS AND METHODS

BSA (essentially fatty acid free) and myoglobin were purchased from Sigma Chemical Co. (St. Louis, MO) and used without further purification. Halothane (2-bromo-2-chloro-1,1,1-trifluoroethane) was purchased from Halocarbon Laboratories (Hackensack, NJ) and passed through an alumina column before use to remove water and thymol. Tritiated water (specific activity = 100 mCi/ml) was from Amersham (Arlington Heights, IL). [^{14}C]Halothane (51 mCi/mmol) from DuPont-NEN (Boston, MA) was diluted immediately in water to a 5 mM solution and reduced specific activity of 3 mCi/mmol. All other chemicals were obtained from Sigma and were of reagent grade.

Photoaffinity labeling

Halothane binding parameters were obtained from competition assays using direct photoaffinity labeling of [^{14}C]halothane (Eckenhoff and Shuman, 1993). Solutions of protein (generally 1 mg/ml) were prepared in deoxygenated phosphate buffer (concentration and pH values given below for individual experiments) and placed in 0.5-cm-path-length 2.0-ml quartz cuvettes, together with increasing concentrations of either unlabeled halothane (0–7 mM) or guanidine hydrochloride (0–3 M) prepared in the same buffers and added from gas-tight Hamilton syringes (Reno, NV). [^{14}C]halothane was also added as a small aliquot from another gas-tight syringe to achieve ~100 μM in the cuvette. Halothane-containing solutions were generally added to the cuvette last, to avoid loss into the gas space. Cuvettes were exposed to the output of a low-pressure Hg(Ar) Oriel pencil calibration lamp at 1 cm distance with constant stirring for 60 s. The cuvette contents were then precipitated with ice-cold 10% trichloroacetic acid (TCA), filtered through Whatman GF/B glass fiber filters, and washed with 8 ml of cold 1% TCA. Incorporated dpm was determined using liquid scintillation after incubation overnight. The effect of guanidine on both the BSA and myoglobin folded fraction was determined with an Aviv 62 CD spectrometer, at a protein concentration of ~10 μM in 10 mM K_2PO_4, pH 7.0.

Hydrogen exchange

Protein was dissolved at 2–10 mg/ml in sodium phosphate buffer (20–100 mM) at pH 9 with 1 M guanidine hydrochloride and incubated with ~5 mCi of ^3H_2O at least 18 h at room temperature to completely equilibrate exchangeable amide hydrogens with solvent hydrogens. Aliquots were removed to determine specific activity of ^{3}H. Free ^{3}HOH was removed from the protein solution with a PD-10 (Sigma) gel filtration column to initiate exchange-out, and this also facilitated a switch to the exchange-out buffer. Because different proteins have different intrinsic hydrogen exchange rates, the exchange-out buffer was adjusted for each protein to allow focus on the slow hydrogens (the last 20 or so to exchange) in a convenient time window (less than 10 h). For BSA, the exchange-out conditions were 100 mM sodium phosphate at pH 7.4 with 1 M guanidine hydrochloride and 38°C. To focus on groups of more rapidly exchanging hydrogens, the intrinsic HX rate was slowed in some exchange-out experiments by reducing the pH to 6.0 or 7.0 and lowering the temperature to 23°C; guanidine was also eliminated in these experiments. Exchange-out conditions for myoglobin were similar to BSA, except that guanidine was excluded and the pH was raised to 8.0. After recovery from the column, the protein solution was immediately transferred to prefilled Hamilton gas-tight syringes (with or without halothane) equipped with repeaters, and aliquots were delivered into ice-cold 10% TCA at timed intervals. The precipitated protein was vacuum filtered through Whatman GF/B filters and washed with 8 ml of ice-cold 1% TCA. The entire precipitation/filtration procedure was routinely accomplished in 10 s or less, and loss 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 (1984). After equilibration of the filters with fluor overnight, retained ^{3}H was determined with scintillation counting. Protein concentration of parallel repeater aliquots was determined by absorbance at 280 nm using extinction coefficients of 45,000 cm^{-1} M^{-1} for BSA and 30,400 cm^{-1} M^{-1} for myoglobin. Small (5–10 μl) samples of the exchange-in solution were precipitated as above to assure equilibration of all exchangeable hydrogens.

Data analysis

Binding parameters were determined from a nonlinear least squares fit of the competition data to sigmoid curves of negative and variable slope using InPlot Prizm (v2.01) software. Global exchange-out curves for large proteins such as BSA result from the exchange of numerous individual or grouped hydrogens, each exchanging with their own time constants. Therefore, as global exchange is a complex multiexponential function with unknown components, we chose not to arbitrarily fit the data to obtain global exchange-out rates. Rather, protection factors for given hydrogens were determined from the data. Assuming horizontal equivalence of hydrogen exchange (the nth hydrogen to exchange is the same with and without anesthetic), protection factor ratios were estimated by dividing the time required for a given hydrogen to exchange under the differing conditions (e.g., with and without anesthetic) and were determined for the last three to five hydrogens in common for the two conditions. Protection factor ratios (PFRs) were then averaged, and $\Delta\Delta G$ was determined using $\Delta\Delta G = -RT\ln(\mathrm{PFR})$.

RESULTS

Bovine serum albumin

Unlabeled halothane competed with label incorporation into BSA with an IC_{50} of 0.2 mM and Hill coefficient of -0.7 (Fig. 1), similar to what we have previously reported for this protein. Apparent dissociation constants (K_D) can be obtained using the following equation:

$$K_I = (IC_{50})/(1 + [[^{14}C]\text{halothane}]/K_D)$$

and when the competing ligands are the same:

$$K_D = IC_{50} - [[^{14}C]\text{halothane}]$$

Therefore, the apparent K_D for halothane photolabeling of BSA is 0.1 mM. The conformation dependence of this specific binding has been suggested by our previous results with different pH values (Eckenhoff and Shuman, 1993) but also shown here by the effect of guanidine. Fig. 2 shows that, although low concentrations of guanidine had a minimal effect on label incorporation, progressively higher concentrations significantly inhibited labeling. The guanidine IC_{50} was 1.8 $\pm$ 0.2 M, slightly less than that causing a 50%

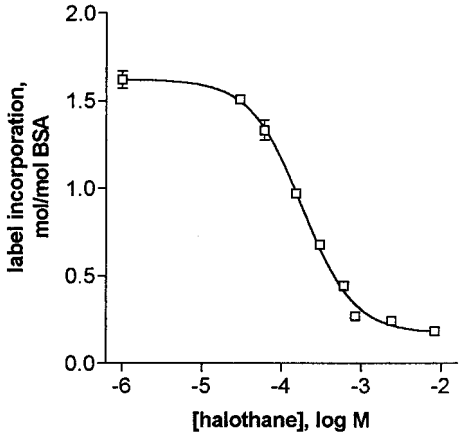

FIGURE 1 Competition curve for [^{14}C]halothane (100 μM) labeling of bovine serum albumin, using unlabeled halothane as a competitor. ~Points are the mean of at least three experiments, with three replicates, and the line is a nonlinear least squares fit to Hill plots with variable negative slope. IC_{50} = 0.18 (95% C.I. = 0.15–0.22); Hill = −1.3 ± 0.12.

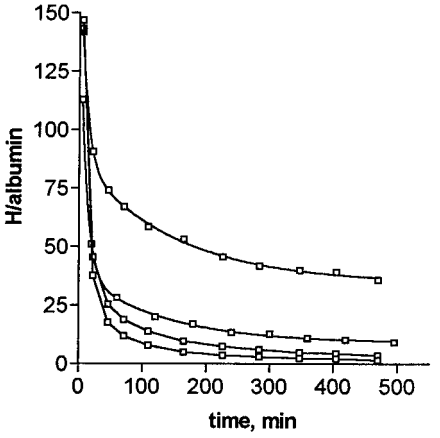

FIGURE 3 Hydrogen-tritium exchange-out from serum albumin at pH 7.4, 1 M guanidine, and 38°C, and the effect of added halothane. From the bottom, the curves are 0 mM, 1 mM, 3 mM, and 10 mM halothane.

loss in helicity as determined with circular dichroism spectroscopy (2.2 ± 0.03 M; Fig. 2.).

Fig. 3 shows ^{3}H exchange-out from BSA in different concentrations of halothane. Halothane decreases the HX rate of these slow amide hydrogens in a concentration-dependent fashion, implying a stabilization of the protein through preferential binding to the native, folded state, consistent with the conformational dependence of binding as determined above (Fig. 2). PFRs and the corresponding free energy changes are given in Table 1. Whereas the calculated $\Delta\Delta G$ values assume that these last 40 BSA hydrogens exchange through the same global unfolding event, they are remarkably consistent with the estimated $\Delta\Delta G$ for halothane stabilization of BSA using DSC (Tanner et al., 1997). By lowering the pH to slow chemical exchange of hydrogens, and excluding guanidine, so that a group of more rapidly exchanging, and presumably more solvent-exposed, BSA hydrogens can be probed, it could be seen that 10 mM halothane significantly accelerated exchange of the group of hydrogens from ~350 to ~170, while producing no significant effect on the group of BSA hydrogens from ~170 to ~120 (Fig. 4). This is in sharp contrast to the effect of this concentration of halothane on the slower exchanging group of amide hydrogens (<100; Fig. 3) and suggests that there are differential intramolecular effects of halothane binding in this protein.

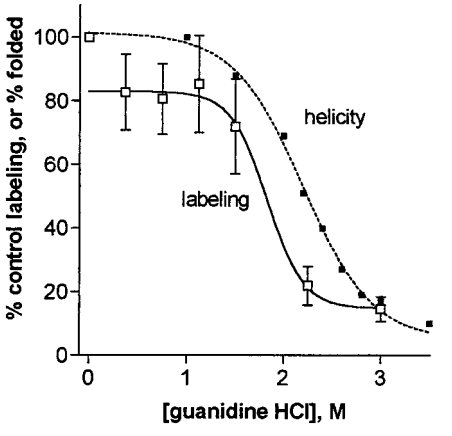

FIGURE 2 Similar to Fig. 1, except guanidine was used as a competing ligand for [^{14}C]halothane photolabeling of BSA (□; fitted IC_{50} = 2.2 ± 0.03 M). Also shown is the percent folded from ellipticity at 222 nm (■; fitted IC_{50} = 1.8 ± 0.1 M).

Myoglobin

As shown in Fig. 5, the labeling of myoglobin by ~100 μM [^{14}C]halothane is at least 50-fold less than that of BSA, but there is some inhibition of label incorporation by high concentrations of unlabeled halothane, suggesting the presence of a weak specific component to the total binding. Thus, although a halothane IC_{50} value for myoglobin cannot be determined with confidence, it is in excess of 10 mM. On the other hand, Fig. 6 shows almost complete inhibition of label incorporation by guanidine concentrations (<0.3M) that produce no change in myoglobin helicity (guanidine IC_{50} of 1.7 + 0.1 M). This is interpreted to indicate that, in contrast to BSA, most halothane binding sites in myoglobin are nonspecific and more solvent-exposed. If one assumes that guanidine unfolds proteins by recruitment of weak

TABLE 1 Protection factor ratios and free energy change

Protein	[Halothane]	Hydrogen range*	PFR#	$\Delta\Delta G$§
BSA	1 mM	4–8	2.0	−0.42
BSA	3 mM	9–13	4.7	−0.96
BSA	10 mM	34–38	27	−2.04
Myoglobin	1 mM	18–20	0.80	+0.14
Myoglobin	10 mM	18–20	0.25	+0.86

*Range of hydrogens for which protection factors were determined.
#Time (min) for control exchange of H above divided by that for halothane.
§In kcal/mol protein.

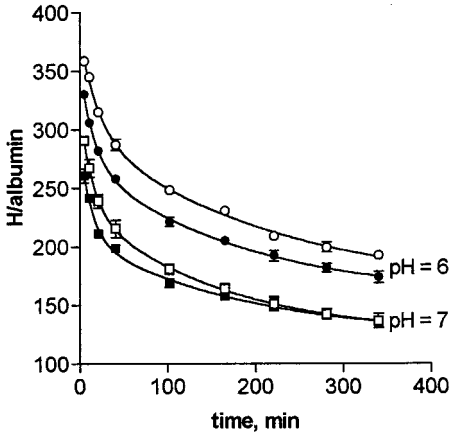

FIGURE 4 Hydrogen-tritium exchange from BSA at pH 6.0 (○ and ●) and pH 7.0 (□ and ■) at 23°C. ○ and □, control; ● and ■, 10 mM halothane. Each set represents three individual experiments, with data points for each group normalized to the average 5-min point. Standard error bars are shown.

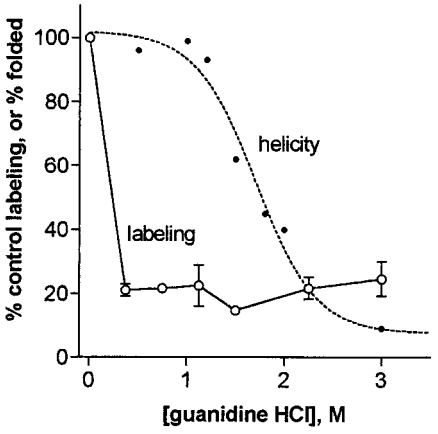

FIGURE 6 Guanidine titration of [^{14}C]halothane photolabeling of myoglobin demonstrates near complete inhibition at [guanidine] < 0.3 M (○), far lower than required for unfolding this protein based on ellipticity at 222 nm (●; guanidine IC_{50} = 1.7 ± 0.1 M).

binding sites in the progressively more unfolded states (Nozaki and Tanford, 1970), competition between guanidine and halothane would imply that the anesthetic is also binding to analogous sites in the unfolded state. Like guanidine, then, one might expect halothane to destabilize myoglobin, which is confirmed by the HX results shown in Fig. 7. Although 1 mM halothane had only a minor effect on exchange-out from myoglobin, 10 mM halothane decreased protection factors for hydrogens 18–20 by approximately fourfold. The exchange of more rapidly exchanging hydrogens (100–20) was also increased by 10 mM hydrogen (data not shown).

DISCUSSION

Photoaffinity labeling clearly distinguished between these two soluble proteins with similar stability, showing at least a 50-fold lower affinity of myoglobin for halothane. Precise stoichiometry is difficult to determine from photoaffinity competition assays, but estimates are possible when using the same ligand as the competitor by recalculating the specific activity of the label at the IC_{50} and correcting the incorporation for this lower specific activity. Thus, for BSA, the stoichiometry at IC_{50} is ~2.7 mol halothane/mol BSA, or an estimated B_{max} of ~5.4 mol/mol. This is consistent with our preliminary DSC results (Tanner et al., 1997), which suggested a lower limit of approximately five sites, and isothermal titration calorimetry results in albumin with a similar halogenated alkane anesthetic, chloroform (Ueda and Yamanaka, 1997), suggesting four higher-affinity sites and several lower-affinity ones. The uncertainty of IC_{50} for myoglobin precludes confidence in a calculated B_{max} value, but data shown in Fig. 4 suggest a stoichiometry of at least 5 mol/mol.

Although the mechanism of guanidine inhibition of photolabeling is not clear at this point, the data are consistent

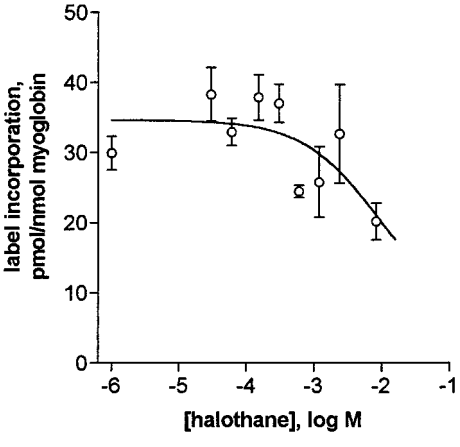

FIGURE 5 Competition curve as in Fig. 1, except for myoglobin. The line represents the best fit, but parameters are not reported because of poor confidence in the 50% inhibition region, and higher halothane concentrations were not used because of solubility limitations and UV-screening effects of high halothane concentrations. Note very low labeling stoichiometry at this low [^{14}C]halothane control concentration (~100 μM).

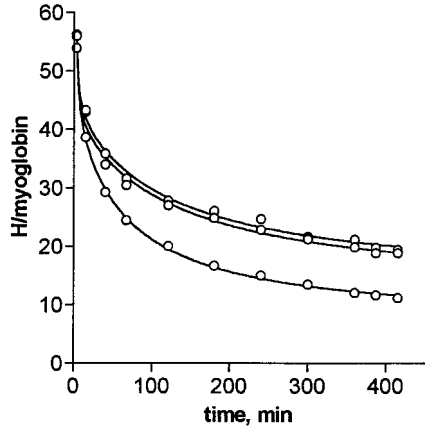

FIGURE 7 Hydrogen-tritium exchange of myoglobin at pH 8 and 38°C and the effect of added halothane. From the bottom, curves are 10 mM, 1 mM, and 0 mM halothane.

with competition at nonspecific binding sites. Although halothane and guanidine clearly have different polar character, nonspecific binding is, by definition, characterized by very low interaction energy and should therefore be relatively nonselective. Guanidine titration of halothane photoaffinity labeling allowed separation of at least two classes of binding sites in these two model proteins, and perhaps a basis for the different binding character. One class of site appears to be freely exposed to solvent guanidine and does not distinguish between solvent halothane and guanidine, and a second group of sites is more protected from solvent guanidine, presumably in internal, hydrophobic environments. As the protein becomes progressively more exposed with the increasing concentrations of guanidine, there is less protection for the hydrophobic environments from the solvent, effectively removing this more specific class of halothane binding site. It is predicted that specific sites are a feature of native tertiary structure, which is likely the basis for the slightly lower guanidine IC_{50} for binding as compared with helicity, a measure of secondary structure. BSA is an example of a protein that contains predominantly this specific class of site. Binding to myoglobin, on the other hand, is entirely lost at a guanidine concentration resulting in no loss of helicity, suggesting that sites are more solvent-exposed. Therefore, these results would appear to implicate hydrophobic cavities as the basis for preferred binding to BSA, essentially an entropic event. Weak electrostatics may also contribute, however, as we have shown that at least two of the halothane binding sites contain tryptophan residues (Johansson et al., 1995; Eckenhoff, 1996), and the van't Hoff relationship for halothane binding to these regions using tryptophan fluorescence quenching yields a ΔH of ~ -1 kcal/mol (Johansson and Eckenhoff, 1997). Thus, the results are most consistent with binding of multiple halothane molecules on these two proteins but at sites of different character and degree of solvent exposure. Furthermore, the clear distinction between the binding character in these proteins gives additional confidence that halothane photolabeling is reliably reporting sites of equilibrium distribution.

The HX results show that this conformation-dependent binding of halothane to protein has clear consequences in terms of overall protein stability as reflected by hydrogen exchange behavior. The protection factor refers to the difference in exchange kinetics of a given population of amide hydrogens in a peptide relative to that of a completely exposed, non-hydrogen-bonded amide hydrogen (half-time of $\sim 10^{-4}$ s at pH 7 and 20°C) and normally ranges over several orders of magnitude. The last hydrogens to exchange are thought to do so through a cooperative and transient but global unfolding process, so native-state protection factors (PF_{Native}) should be proportional to $1/K_{unfold}$. Making allowances for the pH and temperature effects on chemical exchange rates (Englander and Englander, 1984), our results of greater than 500 min for exchange of the last 20 hydrogens of myoglobin at 38°C and pH 8 translate to protection factors of $\sim 10^{10}$, yielding an unfolding ΔG^{o} of $\sim$13 kcal/mol, consistent with previous results in myoglobin (Pace and Vanderburg, 1979). BSA appears to be even more stable, 1 M guanidine being necessary to bring this population of hydrogens into the same time frame. The slowing of hydrogen exchange (increase in protection factors) in BSA confirms preferential binding of halothane to the native, folded conformation of this protein as predicted by the guanidine titration (this study), the effect of pH on halothane binding (Eckenhoff and Shuman, 1993), and the preliminary calorimetry results (Tanner et al., 1997).

Assuming that exposure of the slowly exchanging hydrogens is a result of global unfolding, and that halothane binds predominantly to independent sites of identical affinity in the native form of BSA, then the ratio of protection factors ($PF_{Halothane}/PF_{Control}$ or PFR) for the slowest hydrogens would be predicted to be $(1 + [\text{halothane}]/K_D)^n$, where n is the number of binding sites and K_D is the dissociation constant for halothane binding to native state of BSA. Using our experimentally derived K_D of 0.1 mM and assuming, for the moment, a single site, 10 mM halothane is predicted to produce a 100-fold change in PF, 3 mM halothane $\sim$30-fold, and 1 mM halothane $\sim$10-fold. As the observed changes in PF in this study are all four- to fivefold lower than this, either the actual K_D is higher than 0.1 mM or these BSA hydrogens are exchanging through less than global unfolding events. The discrepancy between measured and predicted changes in PF is made much larger when considering that evidence suggests multiple halothane binding sites. Photolabeling K_D values are indeed an underestimation of true equilibrium values, probably because of irreversible depletion of sites at high unlabeled halothane concentrations. The magnitude could be as large as tenfold, as shown by ^{19}F-NMR spectroscopy (Dubois and Evers, 1992) or tryptophan fluorescence quenching (Johansson et al., 1995) studies, which estimated K_D values for halothane binding to BSA of $\sim$1 mM. Even using this 1 mM K_D value, however, 10 mM halothane should change PF by a factor of 10^5 if all five sites are considered. Although the five BSA halothane binding sites probably have different K_D values, it seems likely that at least a portion of the last 40 hydrogens to exchange out of BSA do so through less than global unfolding events.

Interestingly, halothane has a different influence on the protection of more rapidly exchanging BSA amide hydrogens. In the most rapidly exchanging population probed in this study, we find approximately a twofold decrease in protection factor, implying that microstability, or rapid local fluctuations in at least some regions of BSA, is modulated differently by the anesthetic than macrostability. It is not yet clear whether this represents a large localized change in BSA structure or dynamics on binding halothane or a smaller more widespread influence. This distinction may be important if protein activity correlates better with local, as opposed to global, motions.

On the other hand, myoglobin, also a very soluble carrier protein, binds halothane poorly (as indicated by photolabeling affinity) and in a way that only accelerates HX. This destabilization suggests preferential binding to an unfolded

form of the protein. Whether the preferential binding is to the completely unfolded form of myoglobin or to an intermediate is not yet clear, but our previous results suggest the latter. In poly-(L-lysine), for example, the pH 12 α-helical conformation was photolabeled by halothane to a considerably greater stoichiometry and affinity than the pH 7 random coil form (Eckenhoff and Shuman, 1993), and a chain length dependence to this binding implied the requirement for supersecondary structure in addition to secondary structure (Johansson and Eckenhoff, 1996). Preferential binding to an intermediate implies that, although supersecondary structure is necessary to create a (hydrophobic) binding site, native tertiary structure essentially precludes binding, presumably due to steric and/or dynamic constraints. The probability of such steric constraints to binding in myoglobin is suggested by x-ray crystallographic evidence that places only the smaller anesthetics dichloromethane, cyclopropane, or xenon in the occupied heme cavity (Schoenborn, 1967, 1976; Tilton et al., 1984).

It is as yet difficult to reconcile these results in model proteins with a general model of anesthetic interaction with more functionally relevant proteins, and such attempts are best viewed with caution. Nevertheless, some intriguing observations can be made. For example, pressure (100–200 atm) antagonizes anesthesia (Wann and MacDonald, 1988) and also reduces the stability of most proteins (Weber and Drickamer, 1983), because of a lower apparent molecular volume in the unfolded state. Therefore, at the molecular level, pressure would be predicted to antagonize events resulting in stabilization and enhance destabilization influences, suggesting that stabilizing events in at least some proteins are important in producing the anesthetic state, or at least in reflecting relevant anesthetic-protein interactions. Likewise, decreasing temperature increases volatile anesthetic potency (independent of the effect on solubility) (Franks and Lieb, 1996) and increases protein stability (at least in the 20–40°C range) (Privalov, 1979), also consistent with stabilization being a pharmacologically important mode of anesthetic/protein interaction. Finally, the somewhat higher affinity of the binding interaction resulting in stabilization events (e.g., BSA) is much closer to the concentration required to produce anesthesia in animals (~0.2–0.4 mM), although the necessary magnitudes of stabilization, the domains stabilized, or the proteins stabilized are by no means clear at this point.

Although it may be attractive to speculate that stabilization is involved in the action of anesthetics, it is interesting to note that other attempts to characterize the dynamic consequences of anesthetic-protein interactions have found that anesthetics tend to increase dynamics (Bigelow and Thomas, 1987; Abadji et al., 1994; Cobb et al., 1990). It may be important to point out that these studies were of membrane protein (Ca^{2+} ATPase, nicotinic acetylcholine receptor, and erythrocyte anion exchange protein) and that the time domain examined was considerably faster than probed here. Given that halothane had different effects on different groups of hydrogens in serum albumin, it is possible that these studies in membrane protein reflect local events and that it is not clear in what direction, or even if, global stability was altered.

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REFERENCES

Abadji, V. C., D. E. Raines, L. A. Dalton, and K. W. Miller. 1994. Lipid-protein interactions and protein dynamics in vesicles containing the nicotinic acetylcholine receptor. *Biochim. Biophys. Acta*. 1194: 25–34.

Alifimoff, J. K., and K. W. Miller. 1993. The mechanism of action of general anesthetic agents. *In* Principles and Practice of Anesthesiology. M. C. Rogers, J. H. Tinker, B. G. Covino, and D. E. Longnecker, editors. Mosby, St. Louis. 1034–1052.

Bai, Y., J. S. Milne, L. Mayne, and S. W. Englander. 1994. Protein stability parameters measured by hydrogen exchange. *Proteins.* 20:4–14.

Bigelow, D. J., and D. D. Thomas. 1987. Rotational dynamics of lipid and the Ca-ATPase in sarcoplasmic reticulum. *J. Biol. Chem.* 262: 13449–13456.

Chiou, J-S., and I. Ueda. 1994. Ethanol unfolds firefly luciferase while competitive inhibitors antagonize unfolding: DSC and FTIR analyses. *J. Pharmacol. Biomed. Anal.* 12:969–975.

Cobb, C. E., S. Juliao, K. Balasubramanian, J. V. Staros, and A. H. Beth. 1990. Effects of diethyl ether on membrane lipid ordering and on rotational dynamics of the anion exchange protein in intact human erythrocytes. *Biochemistry*. 29:10799–10806.

Dubois, B. W., S. Cherian, and A. S. Evers. 1993. Volatile anesthetics compete for common binding sites on bovine sertum albumin. *Proc. Natl. Acad. Sci. U.S.A.* 90:6478–6482.

Dubois, B. W., and A. S. Evers. 1992. ^{19}F-NMR spin-spin relaxation (T_2) method for characterizing anesthetic binding to proteins: analysis of isoflurane binding to albumin. *Biochemistry*. 31:7069–7076.

Eckenhoff, R. G. 1996. Amino acid resolution of halothane binding sites in serum albumin. *J. Biol. Chem.* 271:15521–15526.

Eckenhoff, R. G., and H. Shuman. 1993. Halothane binding to soluble proteins determined by photoaffinity labeling. *Anesthesiology*. 79: 96–106.

Englander, S. W., and J. J. Englander. 1994. Structure and energy change in hemoglobin by hydrogen exchange labeling. *Methods Enzymol*. 232: 26–42.

Evers, A. S., B. W. Dubois, and K. E. Burris. 1995. Saturable binding of volatile anesthetics to proteins studied by ^{19}F-NMR spectroscopy and photoaffinity labeling. *Prog. Anesth. Mech.* 3:151–157.

Franks, N. P., and W. R. Lieb. 1984. Do general anaesthetics act by competitive binding to specific receptors? *Nature*. 310:599–601.

Franks, N. P., and W. R. Lieb. 1996. Temperature dependence of the potency of volatile general anesthetics: implications for in vitro experiments. *Anesthesiology*. 84:716–720.

Johansson, J. S., R. G. Eckenhoff, and P. L. Dutton. 1995. Binding of halothane to serum albumin demonstrated using tryptophan fluorescence. *Anesthesiology*. 83:316–324.

Johansson, J. S., and R. G. Eckenhoff. 1996. Minimum structural requirement for an inhalational anesthetic binding site on a protein target. *Biochim. Biophys. Acta*. 1290:63–68.

Johansson, J. S., and R. G. Eckenhoff. 1997. Experimental approaches to the study of volatile anesthetic-protein interactions. *Pharmacol. Rev.* 49:343–367.

Nozaki, Y., and C. Tanford. 1970. The solubility of amino acids diglycine and triglycine in aqueous guanidine hydrochloride solutions. *J. Biol. Chem.* 245:1648–1652.

Pace, C. N., and K. E. Vanderburg. 1979. Determining globular protein stability: guanidine hydrochloride denaturation of myoglobin. *Biochemistry*. 18:288–292.

Privalov, P. L. 1979. Stability of proteins: small globular proteins. *Adv. Protein Chem.* 33:167.

Schoenborn, B. P. 1967. Binding of cyclopropane to sperm whale myoglobin. *Nature*. 214:1120–1122.

Schoenborn, B. P. 1976. Dichloromethane as an antisickling agent in sickle cell hemoglobin. *Proc. Natl. Acad. Sci. U.S.A.* 73:4195–4199.

Tanner, J. W., R. G. Eckenhoff, and P. A. Liebman. 1997. Protein stabilization by an inhalational anesthetic. *Biophys. J.* 72:A417.

Tilton, R. F., I. D. Kuntz, and G. A. Pesko. 1984. Cavities in proteins: structure of a metmyoglobin-xenon complex solved to 1.9 Å. *Biochemistry*. 23:2849–2857.

Ueda, I., and M. Yamanaka. 1997. Titration calorimetry of anesthetic-protein interaction: negative enthalpy of binding and anesthetic potency. *Biophys. J.* 72:1812–1817.

Wann, K. T., and A. G. MacDonald. 1988. Actions and interactions of high pressure and general anesthetics. *Prog. Neurobiol.* 30:271–307.

Weber, G., and H. G. Q. Drickamer. 1983. The effects of high pressure upon proteins and other biomolecules. *Q. Rev. Biophys.* 16:89–112.