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ACCELERATED COMMUNICATION

Do Specific or Nonspecific Interactions with Proteins Underlie Inhalational Anesthetic Action?

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ABSTRACT

To determine whether specific or nonspecific interactions between inhaled anesthetics and proteins are more likely to underlie anesthetic actions, analysis of hydrogen/tritium exchange was used to measure effects on the stability of two model proteins that had been previously shown to bind anesthetics specifically (bovine serum albumin) or only nonspecifically (myoglobin). The data indicated that stabilization of albumin correlated with the potencies of a wide range of anesthetic compounds significantly better than did destabilization of myoglobin. In addition, sensitivity to nonanesthetics, isoflurane stereoselectivity, and temperature and pressure effects all influenced the stabilization of bovine serum albumin, but not the destabilization of myoglobin, in a manner strongly supporting the premise that specific binding interactions with protein targets underlie anesthetic action. These observations significantly increase the likelihood that such interactions can be found and optimized.

Although molecular targets for volatile anesthetic action have yet to be established, the evolving consensus is that these sites are protein in nature. Protein targets generally involve specific sites and interactions with the ligand, but the low affinity and the multiple chemical structures capable of producing anesthesia raise questions regarding the uniqueness or specificity of the interactions. For example, the enzyme firefly luciferase has been termed the best available protein model of anesthetic action, because inhibition of its activity by many different anesthetics quantitatively approximates their pharmacodynamics in mammals (Franks and Lieb, 1984). Experimentally, the inhibition is competitive with luciferin, suggesting that the anesthetics bind in the substrate binding pocket. Also, for this enzyme, IC_{50} values for certain anesthetics have a negative temperature dependence that predicts a small negative enthalpy ($\Delta H = -2$ kcal/mol of anesthetic) for the interaction (Dickinson *et al.*, 1993), which is a characteristic of the electrostatic interactions underlying many specific ligand-protein interactions. However, despite this functional evidence that suggests specific interactions, DSC shows that the same volatile anesthetics destabilize firefly luciferase against thermal denaturation (Chiou and Ueda, 1994). Destabilization by a ligand indicates that the ligand binds preferentially (either at more sites or with higher affinity) to the unfolded (denatured) enzyme or to a partially folded intermediate form of the enzyme. Such effects are characteristic of nonspecific interactions, in that the nonspecific ligand binds weakly to many different groups at the protein/water interface, so that binding becomes a function of ligand concentration and available solvent-exposed protein surface area, which is increased through unfolding events. Therefore, whereas the functional evidence points toward specific interactions between luciferase and anesthetic, the calorimetric results suggest that inhibition is the result of nonspecific interactions, presumably at many allosteric sites that are recruitable through unfolding events. Such extensive study has been applied to few other soluble protein systems, so it remains unclear whether anesthetic actions can be attributed to specific binding interactions (i.e., the binding site is the result of the native tertiary protein structure) or simply nonspecific binding events at exposed protein interfaces.

Volatile anesthetics bind to sites on BSA and HSA that result from the native tertiary structure. Unfolding the proteins by lowering the pH (Dubois and Evers, 1992; Eckenhoff

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ABBREVIATIONS: DSC, differential scanning calorimetry; BSA, bovine serum albumin; F6, 1,2-dichlorohexafluorocyclobutane; HSA, human serum albumin.

and Shuman, 1993; Johansson *et al*., 1995) significantly lowers anesthetic binding and removes the saturable component. DSC and amide hydrogen exchange (Tanner *et al*., 1997; Eckenhoff and Tanner, 1998) show that BSA is substantially stabilized against unfolding by anesthetics. Therefore, by the definition given above, BSA can serve as a reasonable model of specific binding interactions for at least some inhaled anesthetics. Based on the calorimetric results, firefly luciferase might be an appropriate model of nonspecific interactions; however, direct binding studies with this protein have yet to be published, so unambiguous assignment to either the specific or nonspecific category of interaction is not yet possible. We recently showed that metmyoglobin, a 17-kDa soluble protein, binds halothane poorly and without a saturable component (Eckenhoff and Tanner, 1998). Furthermore, guanidine titration of halothane photolabeling of myoglobin suggests that binding is primarily to solvent-exposed surface area, and DSC and amide hydrogen exchange studies show that halothane destabilizes myoglobin (Tanner *et al*., 1997; Eckenhoff and Tanner, 1998). Taken together, these results indicate that myoglobin is a reasonable model of nonspecific interactions with at least one volatile anesthetic.

In an attempt to determine, in a global sense, which of these interactions is more representative of those underlying anesthetic actions at relevant biological targets, the abilities of these two models to satisfy all of the previously introduced criteria for relevance of sites of anesthetic action were examined. These include (a) correlation of activity with anesthetic potency, similar to the many different Meyer-Overton plots (Alifimoff and Miller, 1993), (b) pressure reversal (Wann and MacDonald, 1988), (c) negative temperature dependence, (d) differential effects of isoflurane stereoisomers (Franks and Lieb, 1994), and (e) differential effects of the recently described nonanesthetic cyclobutanes (Kendig *et al*., 1994; Koblin *et al*., 1994). Amide hydrogen/tritium exchange was used to measure the effects of anesthetics and other perturbations on both BSA and myoglobin (Englander and Englander, 1994), because this assay is a very sensitive measure of shifts in the folded/unfolded equilibria and, therefore, the effects of an added ligand on the magnitude of the equilibrium constant. Specific binding to the native tertiary structure shifts the equilibrium toward the native conformer, exposing internal amide hydrogens less often and thus slowing hydrogen exchange with water, whereas nonspecific binding shifts the equilibrium toward less folded conformers, resulting in more rapid exchange of the normally protected amide hydrogens with water hydrogens.

These proteins are not proposed as functionally relevant targets for volatile anesthetics. Like firefly luciferase, BSA and myoglobin serve only as potential models of anesthetic targets. Unlike luciferase, however, these proteins serve as models for a more proximal step in the overall interaction (i.e., binding). If one model fit the criteria better than the other, this would suggest that a given class of binding (specific or nonspecific) might underlie anesthetic action in more relevant targets and perhaps the molecular features responsible for this interaction might be similar to those in the relevant molecular targets. Furthermore, because effects on amide hydrogen exchange are dynamic sequelae of binding that are closely related to and generally indicative of protein stability, a better correlation of the tested criteria with stabilizing or destabilizing influences might also suggest the dynamic consequences of anesthetic binding in relevant molecular targets.

Experimental Procedures

Materials. BSA (essentially fatty acid-free) and myoglobin (Sigma Chemical, St. Louis, MO) were used without further purification. Compounds used are shown in Table 1, with the estimates of anesthetic EC_{50} values (Roth and Miller, 1984) and sources. The purified isoflurane enantiomers were a gift from Anaquest (Murray Hill, NJ), and thiomethoxyflurane was a gift from Dr. Phil Morgan (Case Western Reserve University, Cleveland, OH). Tritiated water (specific activity, 100 mCi/ml) was from Amersham (Arlington Heights, IL). All other chemicals were reagent grade (Sigma). Compounds were prepared by injection of neat liquid into buffer in gas-tight Hamilton syringes containing microstir bars. These syringes were stirred overnight, and concentrations were verified by gas chromatographic analysis of hexane-extracted samples.

Hydrogen exchange. Protein was dissolved at ~10 mg/ml in 0.1 M sodium phosphate buffer, pH 8–9, with 1 M guanidine HCl and were incubated with approximately 5 mCi of ^{3}HOH for at least 18 hr at room temperature. The high pH accelerates amide hydrogen exchange in exposed amide groups (Englander and Englander, 1994), and 1 M guanidine HCl 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 ^{3}H, for the determination of the number of hydrogens/protein molecule. Exchange-out was initiated by removing free ^{3}HOH from the protein solution with a PD-10 gel filtration column (Sigma); this also facilitated a switch to the exchange-out buffer. This buffer was adjusted to focus on the slowly exchanging hydrogens (the last 5–10% to exchange out) in a convenient time period (<7 hr). Therefore, exchange-out conditions were 0.1 M sodium phosphate, pH 7.4, with 0.5 M guanidine HCl at 37° for most experiments. To focus on the exchange of similar groups of hydrogens in the temperature-dependence and pressure experiments, the pH and guanidine HCl concentration were altered as described in the figure legends. After recovery from the PD-10 column, the protein solution was immediately transferred to prefilled Hamilton gas-tight syringes, with or without the test compound at 10 times the "clinical" EC_{50} (Table 1), and aliquots were delivered into 10% trichloroacetic acid at 2° at timed intervals. The precipitated protein was immediately vacuum-filtered through Whatman GF/B filters and washed with 8 ml of 1% trichloroacetic acid at 2°. The entire precipitation/filtration procedure was routinely accomplished in ≤10 sec, and loss of hydrogens in this brief period was excluded as a possibility by

TABLE 1
Investigated compounds, EC_{50} values, and sources

Compound	Approximate EC_{50}[a]	Source
	M	
Thiomethoxyflurane	5×10^{-5}	Dr. P. Morgan, Case Western Reserve University
Methoxyflurane	2×10^{-4}	Abbott Laboratories
Halothane	2×10^{-4}	Halocarbon Laboratories
Isoflurane	3×10^{-4}	Anaquest, Inc.
Enflurane	5×10^{-4}	Anaquest, Inc.
Chloroform	1×10^{-3}	Sigma Chemical Co.
Ethyl chloride	5×10^{-3}	Aldrich Chemical Co.
Dichloromethane	1×10^{-2}	Sigma Chemical Co.
Diethyl ether	2×10^{-2}	Aldrich Chemical Co.
Ethanol	7×10^{-2}	Sigma Chemical Co.
F3[b]	4×10^{-4}	PCR Inc.
F6	NA[c]	PCR Inc.

[a] EC_{50} values from Ref. 15.
[b] F3, 1-chloro-1,2,2-trifluorocyclobutane.
[c] NA, not available.

comparison of retained activity in parallel protein samples run through a second column, according to the technique of Englander and Englander (1994). After overnight equilibration of the filters with fluor, retained ^{3}H (bound to the protein) was determined by scintillation counting. Syringe protein concentrations were 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, filtered, washed, and counted as described above, to ensure equilibration of all exchangeable hydrogens.

Pressure experiments. To test whether the moderate pressures known to antagonize anesthesia (100 atm) influence amide hydrogen exchange in these two proteins, a stainless steel syringe with a threaded plunger and a Teflon seal was fabricated; it was capable of manually generating hydrostatic pressures of >500 atm. Proteins in exchange-out buffer were loaded into the syringe and pressurized to 100 or 200 atm or were loaded into a nonpressurized vessel (controls). Timed aliquots for precipitation were obtained by briefly decompressing the system. Pressure was monitored continuously with a calibrated transducer and varied by <5% of the target pressure.

Data analysis. Protection factors for given hydrogens were determined from the exchange-out curves (Fig. 1). Assuming horizontal equivalence of hydrogen exchange (the n^{th} hydrogen to exchange is the same hydrogen with and without anesthetic, 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 different 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 ratio (*PFr*) values were then averaged, and $\Delta\Delta G$ was determined using the equation $\Delta\Delta G = -RT\ln(PFr)$. $\Delta\Delta G$ represents the change in free energy of the folded state in the presence of the test compound; negative values reflect stabilization (slower exchange) and positive values indicate destabilization (faster exchange).

Results

All compounds tested accelerated the exchange of slowly exchanging hydrogens from myoglobin, and almost all of the compounds tested decelerated exchange of these hydrogens from BSA. Fig. 2 indicates this graphically and also shows that there was a significant relationship, with positive slope, between compound concentration and the $\Delta\Delta G$ for myoglobin. The effects of these equipotent compound concentrations on the $\Delta\Delta G$ for BSA, on the other hand, were roughly equivalent, and the regression line exhibited a slope not different from 0.

Hydrostatic pressure uniformly caused protection factors for slowly exchanging hydrogens to decrease. A pressure of 100 atm produced a $\Delta\Delta G$ of +0.3 kcal/mol of protein for both myoglobin and BSA (Fig. 3A). In some experiments, I combined 200-atm pressure and 6 mM halothane and found that pressure partially reversed the stabilization produced by this relatively high concentration of halothane (Fig. 3B).

The temperature dependence of the interaction of halothane with both myoglobin and BSA was evaluated by comparing $\Delta\Delta G$ values produced by 6 mM halothane at two different temperatures (22° and 37°). To probe the same group of hydrogens at the two temperatures, it was necessary to alter chemical exchange rates by varying the pH, while keeping the guanidine HCl concentration constant. For BSA, at 22° the pH was 7.7 and at 37° it was 6.7. For myoglobin, at 22° the pH was 7.5 and at 37° it was 8.2. Table 2 lists the values obtained for $\Delta\Delta G$ for each protein at each temperature. From these data, $\Delta\Delta S$ was calculated as $(\Delta\Delta G_{37} - \Delta\Delta G_{22})/15°$ and then $\Delta\Delta H$ was calculated as $\Delta\Delta G_{22} + 295(\Delta\Delta S)$. Therefore, as derived from three separate experiments for each protein, $\Delta\Delta H$ was determined to be -8.3 ± 0.3 kcal/mol of BSA and $+2.1 \pm 0.2$ kcal/mol of myoglobin.

The ability of these protein models to distinguish between the enantiomers of isoflurane was evaluated. Stereoisomers were present at 3.5 mM, and the pair was tested with both myoglobin and BSA in three separate experiments. Fig. 4 shows that there was a small but significant difference between the abilities of the isomers to stabilize BSA, with the (+)-isomer being more potent. This difference in $\Delta\Delta G$ reflects a ~25% difference in protection factors. There was no detectable difference between the abilities of the isomers to destabilize myoglobin.

At concentrations of F6 predicted to cause anesthesia, based on lipid solubility (Meyer-Overton rule), it does not (Kendig *et al.*, 1994; Koblin *et al.*, 1994). Initially classified as a nonanesthetic, F6 causes excitement and amnesia (Kandel

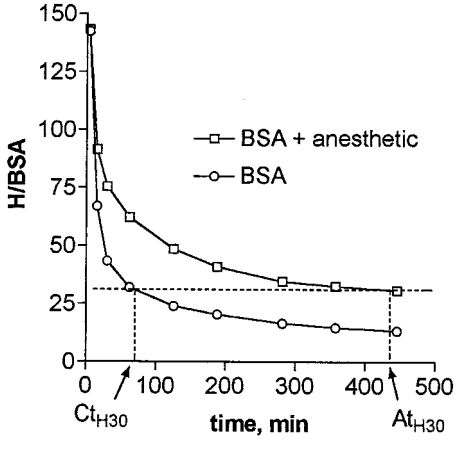

Fig. 1. Typical exchange-out curve for BSA in the presence and absence of an anesthetic. Exchange out is compared by calculating a ratio of the times required for a given slowly exchanging hydrogen (in this case, hydrogen 30) to exchange with and without the anesthetic (At_{H30}/Ct_{H30}) and then determining the change in protein stability as $\Delta\Delta G = -RT\ln(At_{H30}/Ct_{H30})$, where $\Delta\Delta G$ is the change in the free energy of the folded state of BSA in the presence of the anesthetic. This analysis assumes the 30th (for example) hydrogen to exchange is the same with and without anesthetic.

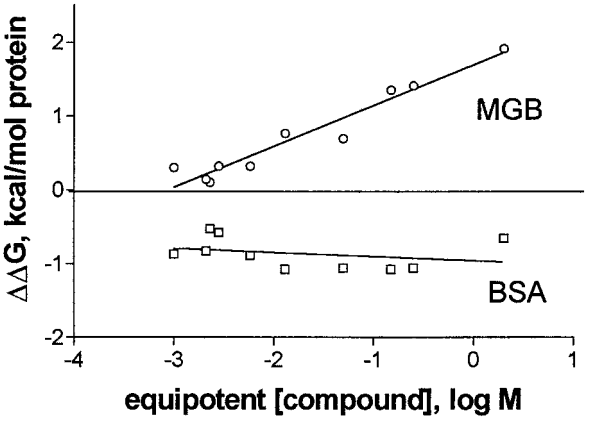

Fig. 2. $\Delta\Delta G$ versus equipotent ($10 \times EC_{50}$) compound concentrations. The compounds destabilized myoglobin (*MGB*) (positive $\Delta\Delta G$ values), yielding a significant positive slope (0.55; 95% confidence interval, 0.44–0.67; $p < 0.0001$) with respect to concentration, whereas they tended to produce similar degrees of stabilization of BSA [i.e., slope not significantly different from 0 (slope, 0.06; 95% confidence interval, −0.21–0.10; $p = 0.42$)].

et al., 1996) and so is now termed a "nonimmobilizer." The maximal aqueous solubility is ~200 μM, at which concentration F6 destabilizes both BSA and myoglobin (Fig. 5). This was the only compound tested that decreased protection factors for the slowly exchanging hydrogens of BSA. An anesthetic but otherwise comparable cyclobutane, 1-chloro-1,2,2-trifluorocyclobutane, stabilized BSA at both low (200 μM) and high (4 mM) concentrations and destabilized myoglobin.

Discussion

Taken together, these results strongly suggest that specific interactions, defined here as preferential interactions between the anesthetic and the native folded state of a protein, underlie anesthetic action. This is opposed to nonspecific interactions [i.e., preferential interactions between the anesthetic and the unfolded (or partially folded) state]. This conclusion is consistent with the notion that specific anesthetic binding sites exist in functionally relevant proteins and that occupancy of these sites stabilizes some conformational but still native state. These results are inconsistent with nonspecific protein interfacial effects underlying important anesthetic protein interactions (Ueda, 1991).

The binding of these small volatile ligands to BSA is likely to occur in the IIA domain (Carter and Ho, 1994), a cavity known to bind a wide variety of hydrophobic polar compounds with varying affinities. This is based on our previous work using photolabeling (Eckenhoff and Shuman, 1993; Eckenhoff, 1996b) and tryptophan fluorescence quenching (Johansson *et al.*, 1995), which demonstrated halothane binding in the vicinity of Trp214 of both BSA and HSA (a conserved residue known to be in the IIA cavity). Several anesthetics compete with halothane binding (Dubois *et al.*, 1993; Eckenhoff and Shuman, 1993; Johansson *et al.*, 1995), supporting the premise that the IIA cavity is a common binding site for such molecules. Halothane also binds with higher affinity in the vicinity of Trp134 in BSA (not present in HSA). Both of these sites are a feature of the native tertiary structure, as demonstrated by the loss of labeling and of fluorescence quenching after unfolding produced by low pH, chaotropic agents, or heat (Dubois and Evers, 1992; Eckenhoff and Shuman, 1993; Johansson *et al.*, 1995; Tanner *et al.*, 1997; Eckenhoff and Tanner, 1998). There are probably additional, lower affinity, anesthetic binding sites in the serum albumins, and all may participate in the observed stabilization. Myoglobin also has two tryptophan residues, but the inability of halothane to quench their fluorescence emission (Johansson *et al.*, 1995) and the inability of photolabeling to reveal saturable binding (Eckenhoff and Tanner, 1998) suggest that their packing leaves insufficient volume to accommodate the anesthetics. Anesthetic binding to myoglobin probably occurs only weakly at protein/water interfaces and is therefore a function of solvent-exposed surface area. This may explain the mechanism of myoglobin destabilization by anesthetics. More binding sites (solvent-exposed surface area) are available in unfolded conformers of myoglobin than in the native state, so in the absence of stronger binding to the native state, as in the case of BSA, the equilibrium is shifted toward the more dynamic unfolded states.

Similarly, destabilization of BSA by F6 suggests that this cyclobutane binds preferentially to unfolded BSA. However, a recent report (Forman and Raines, 1998) showed that F6 quenched HSA tryptophan fluorescence (arising from the conserved tryptophan in the IIA cavity), strongly suggesting F6 binding in the IIA cavity, which is a feature of tertiary structure. These apparently disparate results may be reconciled by realizing the polar nature of the IIA cavity and the fact that F6 has only a very small permanent dipole moment, implying weaker binding, compared with that of the anesthetics. Being the most hydrophobic of the compounds tested (Kendig *et al.*, 1994), F6 may bind to more sites on unfolded BSA conformers (more exposed hydrophobic residues), rather than to only the one on the folded conformer that it shares with the other compounds. The total result of binding to all of these sites is a shift in the equilibrium toward the more dynamic unfolded states (i.e., destabilization). 1-Chloro-1,2,2-trifluorocyclobutane, having a larger permanent dipole and being less hydrophobic, binds preferentially to the specific site, so that the equilibrium is shifted more toward the less dynamic native state. Regardless of the mechanism of F6 destabilization, this result with a nonanesthetic compound is entirely consistent with the hypothesis that preferential binding to native states underlies anesthetic action.

The (+)-enantiomer of isoflurane has been shown to be approximately 20% more potent in intact animals (Franks and Lieb, 1994; Lysko *et al.*, 1994; Eger *et al.*, 1997) and in several *in vitro* systems (Harris *et al.*, 1994; Pohorecki *et al.*, 1994), compared with the (−)-enantiomer. In fact, we and others have reported that the (+)-isomer binds to BSA with slightly higher affinity, compared with the (−)-isomer (Eckenhoff and Shuman, 1993; Xu *et al.*, 1996). Therefore, it

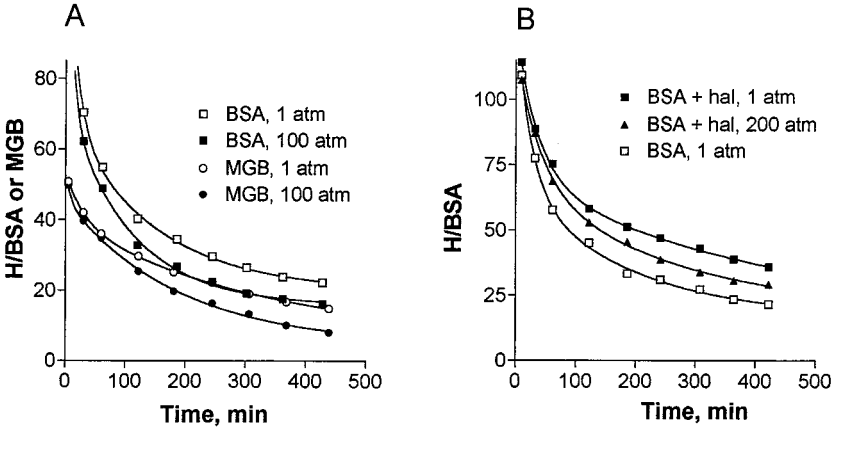

Fig. 3. Pressure effects on hydrogen exchange. A, Effect of 100 atm on hydrogen/tritium exchange in both BSA and myoglobin (*MGB*). The two proteins were destabilized to similar extents (see text). B, Results showing that the stabilizing effect of 6 mM halothane (*hal*) ($\Delta\Delta G = -0.5$ kcal/mol) was partially reversed (by 0.25 kcal/mol) by 200 atm of pressure.

would be predicted that, if stabilization is the result of preferential binding to the native state, then BSA should distinguish between these otherwise identical compounds, in a direction similar to that observed in previous studies. Both the direction and the magnitude of difference between the isomers matched both the BSA labeling results and the *in vivo* potency results remarkably well. Given the range of structures accommodated by the IIA binding site, even this minor degree of selectivity is somewhat surprising. The molecular basis for improved binding of (+)-isoflurane to so many different and general targets is not clear at this point.

The potency of most inhaled anesthetics increases with lower body temperature, an observation that is mostly explained by an increase in aqueous solubility (Antognini, 1993). However, even when results are corrected for solubility changes, a small negative temperature dependence is apparent (Franks and Lieb, 1996). This negative temperature dependence has also been observed in some *in vitro* systems, most notably with the enzyme firefly luciferase (Dickinson *et al.*, 1993). Anesthetic inhibition of this enzyme at different temperatures allows the determination of a ΔH value of approximately −2 kcal/mol of anesthetic, suggesting that, in addition to the hydrophobic effect, electrostatic features may contribute to the binding/inhibition. We have shown that halothane binding to BSA is also characterized by a $-\Delta H$ value of approximately 2 kcal/mol of anesthetic (Johansson and Eckenhoff, 1998); accordingly, in this study I find that stabilization of BSA is slightly more effective at lower temperature. The ΔH of −8 kcal/mol of BSA translates to approximately −1.6 kcal/mol of anesthetic, assuming five occupied binding sites at 6 mM halothane. Destabilization of myoglobin, on the other hand, has a slight positive temperature dependence, which is more consistent with strictly nonspecific hydrophobic interactions and inconsistent with *in vivo* data.

Hydrostatic pressure on the order of 100 atm antagonizes anesthetic action (Wann and MacDonald, 1988), and higher pressure destabilizes proteins because the ΔV of folding is generally positive (Weber and Drickamer, 1983). The primary question addressed here was whether only 100 atm could produce a change in stability of the same direction and magnitude, compared with the anesthetics. In addition to verification of this, the combination of anesthetic and pressure demonstrated the expected antagonism. This further establishes the consistency of binding/stabilization of BSA as a reasonable model of the interactions underlying anesthetic action and also suggests that stabilization itself could be an important underlying mechanism of anesthetic-induced protein dysfunction. Stabilization implies a decrease in protein dynamics on some time scale, and the dynamic behavior of protein is considered to be an important prerequisite for function. For example, mutations in T4 lysozyme that rendered it more stable decreased the enzymatic activity (Lim *et al.*, 1994). Also, lowered temperature (in the 20–40° range) is known to decrease both the dynamics and activities of many proteins. On the other hand, increases in protein dynamics could also cause dysfunction through depopulation of the native folded state (if that state is required for activity). Of note, all studies of anesthetic effects on the dynamics of membrane proteins have noted increases (Bigelow and Thomas, 1987; Cobb *et al.*, 1990; Abadji *et al.*, 1994), although generally in a much faster time domain than probed in this study. It is possible that anesthetics could induce opposite effects on the dynamics in different time domains in the same protein. For example, we recently found that halothane slowed the exchange of slowly exchanging hydrogens in BSA (as shown also here) while accelerating the exchange of more rapidly exchanging hydrogens. Such behavior could underlie the now well established potentiation of the activity of some proteins, particularly membrane-associated receptors and ion channels (Franks and Lieb, 1994). It is possible that recent successes, using mutagenesis approaches, in uncovering residues that control the anesthetic sensitivity of certain receptor/channel complexes (Forman *et al.*, 1995; Mihic *et al.*, 1997) actually indicate modulation of the stability of a functionally important conformation. Small changes in the free-energy difference ($\Delta\Delta G$) between conformational states of

TABLE 2
Free-energy parameters

Protein	$\Delta\Delta G$ at 22°	$\Delta\Delta G$ at 37°	$\Delta\Delta H$ at 22°
	kcal/mol of protein	*kcal/mol of protein*	*kcal/mol of protein*
BSA	−1.53 + 0.07	−1.17 + 0.05	−8.3 + 0.3
Myoglobin	0.61 + 0.05	0.75 + 0.05	2.1 + 0.2

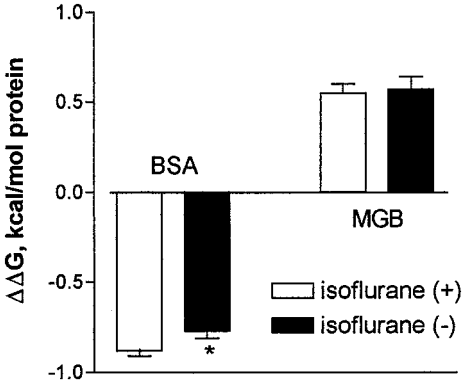

Fig. 4. Evidence that isoflurane enantiomers produce slightly but significantly ($p < 0.01$) different degrees of stabilization of BSA, with no detectable differences in the destabilization of myoglobin (*MGB*). *, $p < 0.01$.

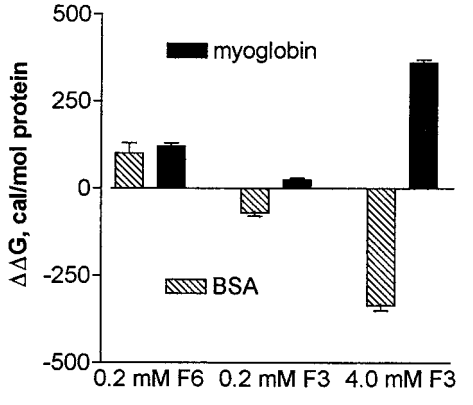

Fig. 5. Evidence that the nonanesthetic F6 was the only compound tested that destabilized BSA to a similar degree, compared with myoglobin. Because the solubility limit of F6 precluded the use of concentrations above 0.2 mM, the effects of F6 were compared with those of 0.2 and 4.0 mM (10 × EC_{50}) 1-chloro-1,2,2-trifluorocyclobutane (*F3*). In both cases, 1-chloro-1,2,2-trifluorocyclobutane stabilized BSA and destabilized myoglobin, by the amounts shown. The $\Delta\Delta G$ value for 4 mM 1-chloro-1,2,2-trifluorocyclobutane was in the same range as that produced by the other compounds at this 10 × EC_{50} concentration.

these proteins could have considerable effects on the abilities of anesthetics to bind to and stabilize them, given the high likelihood of multiple binding sites on such large heterooligomeric protein complexes (Eckenhoff, 1996a).

Concentrations of the compounds used in this study are at least 10-fold higher than those achieved at minimal alveolar concentrations (to produce 50% loss of motor responses to noxious stimulation in animals), for signal/noise purposes. Although some may think that effects measured at such concentrations are unrelated to those that occur at clinical concentrations, the following must be considered. (a) I am proposing not that the magnitude of effects measured here is somehow responsible for anesthesia but, rather, that the type or class of interaction and its direction is related to that in relevant targets. (b) Anesthesia represents a continuum, and loss of a motor response to pain is simply a point on this continuum, rather than representing a saturable maximal phenomenon; a progression of reversible *in vivo* effects can be measured up to at least 3 times the minimal alveolar concentration. (c) Because a binding interaction is being examined, it is important to note that occupancy of binding sites is only rarely related linearly to functional effects. Therefore, clinical EC_{50} and K_d values would not be expected to match; the K_d values for even high affinity ligands are often $\geq$1 order of magnitude higher than clinical EC_{50} values (Eckenhoff and Johansson, 1997). It is not yet clear, because of the paucity of direct binding studies, whether any protein sites can bind these small ligands with K_d values approaching clinical EC_{50} values.

In summary, I used hydrogen/tritium exchange to measure the effects of a variety of anesthetic ligands on the unfolding dynamics of BSA and myoglobin. I found that a variety of criteria associated with anesthetic actions correlated with stabilization of BSA and not destabilization of myoglobin, lending strong support for the notion that specific binding interactions underlie anesthetic action. However, the fact that a protein of clear functional irrelevance can satisfy these criteria so well should raise questions regarding the uniqueness of sites in relevant targets. Despite the implication that functionally important binding sites (and therefore targets) could be widespread, the observation that specific binding interactions underlie action significantly increases the chances that such interactions can be found, manipulated, and optimized.

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