Bound Volatile General Anesthetics Alter Both Local Protein Dynamics and Global Protein Stability

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Background: Recent studies have demonstrated that volatile general anesthetic agents such as halothane and isoflurane may bind to discrete sites on protein targets. In the case of bovine serum albumin, the sites of halothane and chloroform binding have been identified as being located in the IB and IIA subdomains. This structural information provides a foundation for more detailed studies into the potential mechanisms of anesthetic action.

Methods: The effect of halothane and isoflurane and the non-immobilizer 1,2-dichlorohexafluorocyclobutane on the mobility of the indole ring in the tryptophan residues of albumin was investigated using measurements of fluorescence anisotropy. Myoglobin served as a negative control. In addition, the effect of bound anesthetic agents on global protein stability was determined by thermal denaturation experiments using near-ultraviolet circular dichroism spectroscopy.

Results: The fluorescence anisotropy measurements showed that halothane and isoflurane decreased the mobility of the indole rings in a concentration-dependent manner. The calculated dissociation constants were 1.6 ± 0.4 and 1.3 ± 0.3 mM for isoflurane and halothane, respectively. In contrast, both agents failed to increase the fluorescence anisotropy of the tryptophan residues in myoglobin, compatible with lack of binding. The nonimmobilizer 1,2-dichlorohexafluorocyclobutane caused no change in the fluorescence anisotropy of albumin. Binding of the anesthetic agents stabilized the native folded form of albumin to thermal denaturation. Analysis of the thermal denaturation data yielded dissociation constant values of 0.98 ± 0.10 mM for isoflurane and 1.0 ± 0.1 mM for halothane.

Conclusions: Attenuation of local side-chain dynamics and stabilization of folded protein conformations may represent fundamental modes of action of volatile general anesthetic agents. Because protein activity is crucially dependent on inherent flexibility, anesthetic-induced stabilization of certain protein conformations may explain how these important clinical agents change protein function. (Key words: Anesthetic mechanisms; anesthetic–protein interaction; fluorescence.)

THE molecular mechanisms of volatile general anesthetic action remain to be defined.[1] A major obstacle has been the lack of structural information on anesthetic–protein complexes. This is related, in part, to the fact that the *in vivo* sites of action are unknown, although a growing body of evidence implicates the γ-aminobutyric acid A and *N*-methyl-D-aspartate glutamate receptor complexes as playing important roles.[2,3] Further, the unique pharmacology of these agents, characterized by relatively weak binding energetics—translating into rapid dissociation from the binding site, has prevented the use of traditional radioligand approaches to defining binding sites on proteins. In recent years, techniques based on ^{19}F-nuclear magnetic resonance (NMR) spectroscopy,[4] photoaffinity labeling,[5] and fluorescence spectroscopy,[6] which directly monitor the binding of volatile general anesthetic agents to protein targets, have become available. These direct binding studies have set the stage for more detailed biophysical analyses of anesthetic effects on proteins, potentially providing insight into mechanisms of anesthetic action.

Investigations on the mammalian protein bovine serum albumin (BSA), used as a model system, indicate that volatile general anesthetic agents bind to subdomains IB and IIA in close proximity to the two tryptophan residues Trp134 and Trp212.[6-8] Albumin is composed of three homologous domains (I, II, and III), each of which is divided into three subdomains (A, B, and C) for purposes of discussing the location of ligand binding sites.[9] The more water-exposed Trp134 site (in subdomain IB) displays approximately a threefold higher affinity for halothane compared with the Trp212 site (in subdomain IIA).[6] Armed with this initial structural information, it is possible to use well-established biophysical approaches

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Fig. 1. (*A*) Peptide backbone showing potential rotations about the C$^\alpha$-N and C$^\alpha$-C' bonds, characterized by the dihedral angles ϕ and ψ, respectively. (*B*) Tryptophan side chain showing possible rotations about the C$^\alpha$-C$^\beta$ and C$^\beta$-C$^\gamma$ bonds, characterized by the dihedral angles χ^1 and χ^2, respectively. (This figure was generated using Chem3D Plus 3.0 [Cambridge Scientific Computing, Inc., Cambridge, MA] and Canvas 3.5.4 [Deneba Systems, Inc., Miami, FL]. Modified from Demchenko.[13])

One way to quantify protein dynamics (on the nanosecond time scale) is to measure the fluorescence anisotropy[14] of the two tryptophan residues in BSA and to determine the effect of added anesthetic agent. The fluorescence anisotropy is a measure of the average angular displacement (*i.e.*, the mobility) of the indole side chains occurring during the absorption and subsequent emission of a photon.[14] Such alterations in side-chain mobility after anesthetic complexation may represent one of the fundamental molecular mechanisms whereby anesthetic agents ultimately alter protein function. In addition, anesthetic binding may alter protein stability[15,16] by perturbing the equilibrium between the native folded form of the protein target and various partially or fully unfolded conformations. This possibility was examined by thermally denaturing BSA in the absence and presence of the volatile general anesthetic agents halothane and isoflurane. Again, the results of these experiments suggest a basic mechanism whereby a bound anesthetic molecule might alter protein function by decreasing the flexibility of the protein.

Materials and Methods

Materials

Halothane (2-bromo-2-chloro-1,1,1-trifluoroethane) was obtained from Halocarbon Laboratories (Hackensack, NJ). The thymol preservative present in the commercial halothane was removed with an aluminum oxide column.[6] Isoflurane (1-chloro-2,2,2-trifluoroethyl difluoromethyl ether) was purchased from Ohmeda PPD, Inc. (Liberty Corner, NJ). The nonimmobilizer 1,2-dichlorohexafluorocyclobutane was obtained from PCR Incorporated (Gainesville, FL). Fatty acid–free BSA (lot 119F9306) and myoglobin (lot 56F9306, from horse skeletal muscle) were purchased from Sigma Chemical Co. (St. Louis, MO). Guanidinium chloride (8.0 M) was obtained from Pierce (Rockford, IL). All other chemicals were reagent grade.

The buffer used for all experimentation was 130 mM sodium chloride, 20 mM sodium phosphate, *p*H 7.0. Protein solutions were equilibrated with halothane and isoflurane in gas-tight Hamilton (Reno, NV) syringes. Anesthetic-equilibrated protein was diluted with predetermined volumes of plain protein (not exposed to anesthetic agent but otherwise treated in the same manner) to achieve the final anesthetic concentrations shown in figures 2, 4, 8, and 10. Equilibration of BSA with the nonimmobilizer 1,2-dichlorohexafluorocyclobutane was performed in the same manner.

to probe the changes that occur in the protein conformation and dynamics after anesthetic binding. Earlier studies have indicated that anesthetic binding to proteins does not cause any detectable change in protein secondary structure,[6,8,10,11] suggesting that anesthetic binding is associated with more subtle effects on protein structure, presumably at the tertiary level, or perhaps by altering protein dynamics.

The rigid three-dimensional crystal structure of a protein represents a time-averaged equilibrium conformation. Proteins at physiologic temperatures in solution, however, are in reality dynamic entities, with the backbone and side chains in continuous motion.[12] Therefore, there is considerable internal flexibility around bonds between atoms because of random thermal motion as shown in figure 1, with motion being constrained and modulated by neighboring residues, or perhaps by the binding of ligands.

Bovine serum albumin and myoglobin were used without further purification (purity >95% by electrophoresis). Myoglobin contains two tryptophan residues, at positions 7 and 14.[17] Protein concentrations were determined as described[6] after solutions were passed through 0.2 μm nylon syringe filters (Gelman Sciences, Ann Arbor, MI).

Measurements of Fluorescence Anisotropy

The fluorescence anisotropy of BSA (5 μM) was measured at 360 nm (bandwidth 8 nm) after selective excitation of tryptophan residues at 295 nm (bandwidth 2 nm) with a xenon arc lamp on a K2 multifrequency cross-correlation phase and modulation spectrofluorometer (ISS Inc., Champaign, IL), equipped with Glan-Thompson prism polarizers. A 320-nm emission cut-on filter was used to minimize the transmission of scattered excitation light. Anisotropy (*r*) was defined as

$$r = (I_{\parallel} - I_{\perp})/(I_{\parallel} + 2I_{\perp}) \quad (1)$$

where $I_{\parallel}$ and $I_{\perp}$ are the emission intensities polarized parallel and perpendicular to the excitation, respectively.[14] During these conditions, grating (g) values, which correct for emission monochromator polarization effects,[18] ranged from 0.99–1.01. Polarizer alignment was verified[14] with a dilute solution of glycogen in deionized water, yielding an *r* value of 0.98–1.00 (n = 4). The temperature of the cell holder was controlled with a Haake F3 (Berlin, Germany) waterbath.

The concentration dependence of the anesthetic-induced fluorescence anisotropy change was fit using the following relationship:

$$r = r_1 + (r_2 \cdot [A])/(K_d + [A]) \quad (2)$$

where *r* is the measured fluorescence anisotropy, r_1 is the fluorescence anisotropy in the absence of added anesthetic agent, r_2 is the additional limiting fluorescence anisotropy at an infinite anesthetic concentration, [A] is the anesthetic concentration, and K_d is the average dissociation constant for the anesthetic–protein interaction.

Circular Dichroism Spectroscopy

Steady-state near-ultraviolet spectra (240–330 nm) were recorded with a Model 62 DS spectropolarimeter (Aviv, Lakewood, NJ), using 10-mm path length quartz cells, sealed with Teflon stoppers. The cell holder was temperature controlled at 25 ± 0.1°C. The bandwidth

was 1.00 nm, with a scan step of 0.5 nm and an average scan time of 3.0 s.

Thermal denaturation of albumin (22 μM) was followed by circular dichroism (CD) spectroscopy, measuring the ellipticity at 270 nm (Θ_{270nm}) as a function of temperature. The Θ_{270nm} was measured at 1° intervals over the temperature range 50–75°C. The bandwidth was 1.00 nm, temperature equilibration time was 2 min, and the measurement time was 10 s.

For each run, the ellipticity (Θ) *versus* temperature (T) was fit using the relationship:

$$\Theta = [E_n + m_n \cdot T + (E_u + m_u \cdot T) \cdot \exp(\Delta H \cdot \{T/T_m - 1\}/RT)]/[1 + \exp(\Delta H \cdot \{T/T_m - 1\}/RT)], \quad (3)$$

where R is the gas constant (1.987 cal/mol K) and T and T_m (defined later) are in kelvins. We thus obtained best fit values for the six parameters E_n, m_n, E_u, m_u, ΔH, and T_m. This formula describes a standard model for protein unfolding.[19] In this model, the ellipticity at any temperature is given by $\Theta = \Theta_n f_n + \Theta_u f_u$, where Θ_n and Θ_u represent the ellipticity of the native and unfolded states, respectively, and f_n and f_u represent the fraction of protein in the native and unfolded states, respectively ($f_n + f_u = 1$). At low temperatures, in the pretransition region, f_u is close to zero, and $\Theta \approx \Theta_n$, which is assumed to be a linear function of temperature ($\Theta_n = E_n + m_n \cdot T$). At high temperatures, in the posttransition region, f_u is close to 1, and $\Theta \approx \Theta_u$, which also is assumed to be a linear function of temperature ($\Theta_u = E_u + m_u \cdot T$). In the intermediate transition region, f_u varies with temperature such that the equilibrium constant for protein unfolding, $K = f_u/f_n$, satisfies the relationship:

$$K = \exp(\Delta H \cdot \{T/T_m - 1\}/RT). \quad (4)$$

Equation 4 describes an equilibrium with midpoint temperature T_m (at which point K = 1; *i.e.*, $f_u = f_n = 0.5$), and $-\Delta H/R$ is the slope of the van't Hoff plot (lnK *vs.* 1/T). The enthalpy change for protein unfolding (ΔH) describes how sharply the unfolding equilibrium varies with temperature. Thus, for each experiment, a single fitting determined (1) the pre- and posttransition baseline values for Θ_n and Θ_u, so the fraction unfolded (f_u) at each temperature could be calculated from the data using the formula $f_u = (\Theta - \Theta_n)/(\Theta_u - \Theta_n)$,[19] and (2) the parameters T_m and ΔH for determining the unfolding equilibrium constant at any temperature using equation 4.

The dependence of T_m on anesthetic concentration, in conjunction with a value for ΔH, furnishes information
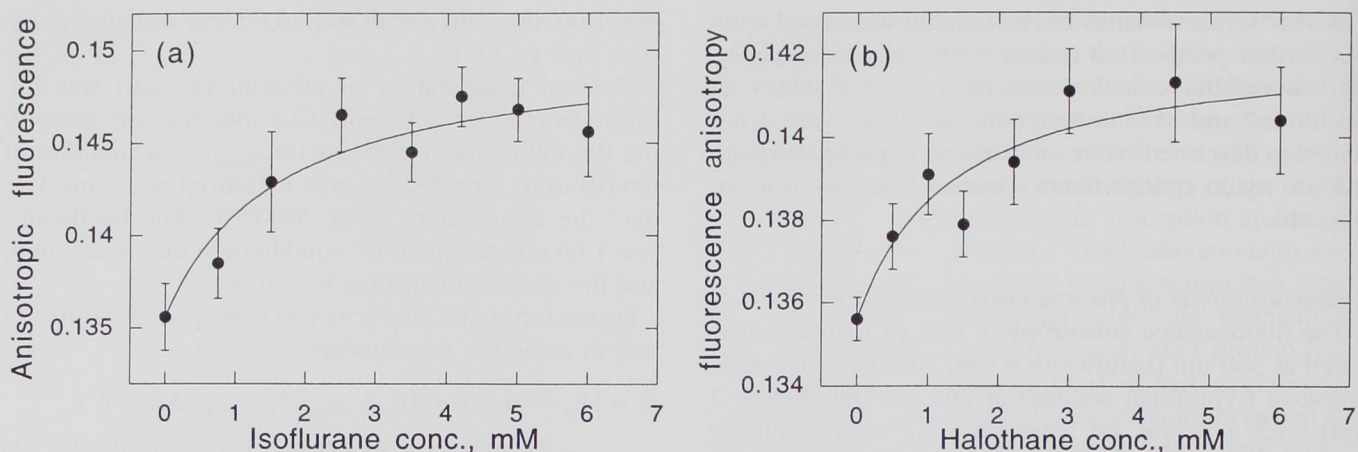
Fig. 2. (A) Tryptophan fluorescence anisotropy of BSA (5 μM) as a function of added isoflurane concentration. (B) Effect of halothane on the tryptophan fluorescence anisotropy of BSA (5 μM). Data points are the means, and error bars are the SEM of 16 different measurements. The lines through the data points were generated using equation 2.

about anesthetic binding parameters. We denote the unfolding equilibrium constant and midpoint temperatures in the absence of anesthetic agent by T_{m0} and K_0, and those in the presence of anesthetic by T_m and K_{app}, in concordance with the terminology used previously.[20] The normalized constant was calculated from the T_m and ΔH results using the equation

$$\ln(K_0/K_{app}) = \Delta H(1/T_{m0} - 1/T_m)/R, \quad (5)$$

which follows from equation 4 evaluated at a temperature halfway between T_{m0} and T_m. Assuming that the native state of BSA has a single binding site for anesthetic agent A with dissociation constant K_d, and the unfolded state lacks this binding site, then the normalized unfolding equilibrium constant will be

$$K_0/K_{app} = (1 + [A]/K_d). \quad (6)$$

That is, the slope of a plot of K_0/K_{app} versus [A] gives the dissociation constant of the anesthetic–protein complex.

Gas Chromatography

Buffer concentrations of halothane, isoflurane, and 1,2-dichlorohexafluorocyclobutane were determined using gas chromatography on an HP 6890 Series instrument (Hewlett Packard, Wilmington, DE), as described.[8,11,21]

Curve Fitting and Statistics

Best fit curves were generated with the KaleidaGraph (Abelbeck Software, Reading, PA) program, and Micro-Cal Origin 2.9 (MicroCal Software, Inc., Northampton, MA). Data are expressed as mean ± SEM.

Results

Changes in Bovine Serum Albumin Fluorescence Anisotropy Associated with Anesthetic Binding

Earlier work using steady-state and time-resolved fluorescence measurements[6,8] and direct photoaffinity labeling[7] has implicated the IB and the IIA subdomains of BSA as volatile anesthetic binding sites. The effect of binding of halothane and isoflurane to the IB and IIA sites in BSA, each of which contains a single tryptophan residue, was examined using fluorescence anisotropy measurements. Figure 2 shows that both anesthetic agents cause a concentration-dependent increase in the tryptophan fluorescence anisotropy of the protein. For both agents, the data have been fit using equation 2, yielding K_d values of 1.6 ± 0.4 and 1.3 ± 0.3 mM for isoflurane and halothane, respectively. Degassing of samples with nitrogen to remove added anesthetic agent resulted in a return of the tryptophan fluorescence anisotropy to control values. Serial dilution of the protein causes no change in the fluorescence anisotropy (although there is a progressive linear change in the steady-state fluorescence intensity), indicating that turbidity-induced light scattering is negligible over the protein concentration range 0.5–10.0 μM and is not responsible for the observed increase in the fluorescence anisotropy in the presence of the volatile anesthetic agents (data not shown).

Effect of the Nonimmobilizer 1,2-Dichlorohexafluorocyclobutane on Bovine Serum Albumin Fluorescence Anisotropy

Polyhalogenated molecules that fail to act like conventional volatile general anesthetic agents have been intro-
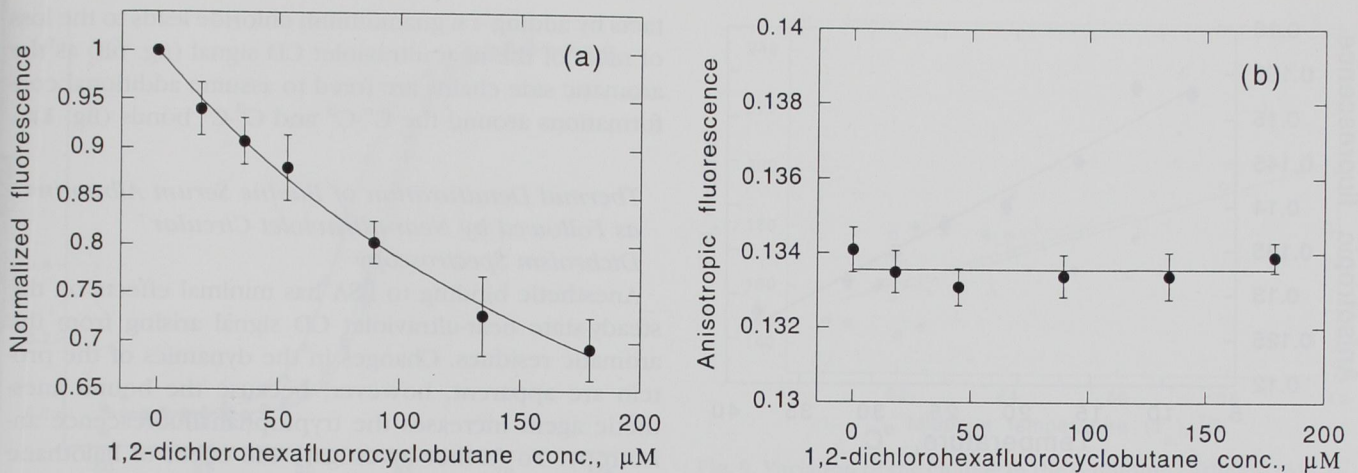
Fig. 3. (*A*) Quenching of BSA (5 μM) tryptophan fluorescence as a function of 1,2-dichlorohexafluorocyclobutane. The curve through the data points was generated using the equation F = 1-(Q_{max} · [1,2-dichlorohexafluorocyclobutane])/(K_d + [1,2-dichlorohexafluorocyclobutane]), where F is the measured steady-state fluorescence; Q_{max} is the maximum fluorescence that can be quenched; and K_d is the dissociation constant for the binding of 1,2-dichlorohexafluorocyclobutane to BSA, as described.[6] The extrapolated Q_{max} is 0.76 ± 0.11, and the K_d is 260 ± 50 μM. Data points are the means of three experiments. Error bars represent the SD. (*B*) Tryptophan fluorescence anisotropy of BSA (5 μM) as a function of added 1,2-dichlorohexafluorocyclobutane concentration. Data points are the means, and error bars are the SEM of nine different measurements.

duced recently[22] and are termed nonimmobilizers. These compounds may serve as tools to test the utility of various model systems currently in use to understand mechanisms of anesthetic action. One example of a nonimmobilizer is 1,2-dichlorohexafluorocyclobutane.[22] This compound recently was shown to bind to human serum albumin[23] using steady-state tryptophan fluorescence measurements to determine binding.[6] Figure 3A shows the effect of 1,2-dichlorohexafluorocyclobutane on BSA steady-state tryptophan fluorescence. There is a concentration-dependent quenching associated with binding of the nonimmobilizer to BSA, with a maximum quenching of 31 ± 3% (n = 3) of the tryptophan fluorescence, at a saturating aqueous concentration of 1,2-dichlorohexafluorocyclobutane of 180 ± 30 μM (n = 8). Figure 3B shows that binding of the nonimmobilizer 1,2-dichlorohexafluorocyclobutane to BSA is not associated with any apparent change in the fluorescence anisotropy of the tryptophan residues.

Effect of Anesthetic Agents on Myoglobin Fluorescence Anisotropy

Experiments were performed with myoglobin to test the specificity of the anesthetic-induced change in the tryptophan fluorescence anisotropy of albumin. Myoglobin also contains two tryptophan residues but has been shown previously not to bind halothane.[6,24] Figure 4 shows the lack of effect of halothane and isoflurane on myoglobin tryptophan fluorescence anisotropy. There-

fore, in contrast to what is observed with albumin, the anesthetic agents have no effect on the myoglobin tryptophan fluorescence anisotropy, indicating an absence of binding to the native, folded form of myoglobin.

Effect of Temperature on Protein Tryptophan Fluorescence Anisotropy

Induced hypothermia has been shown to decrease anesthetic requirements.[25] The effect of temperature on
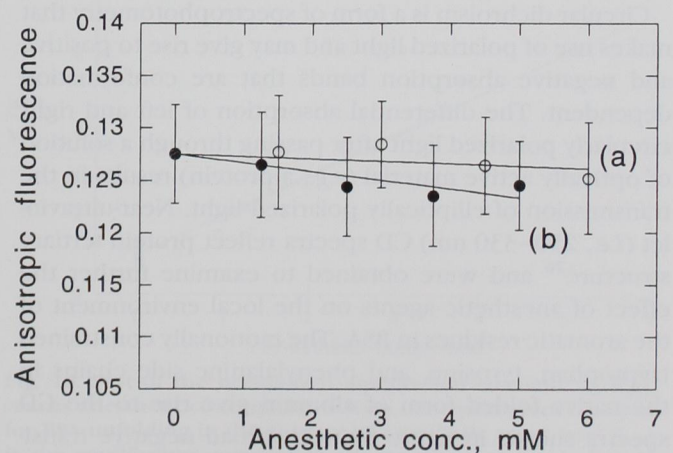

Fig. 4. (*A*) Tryptophan fluorescence anisotropy of myoglobin (4 μM) as a function of added halothane concentration. (*B*) Effect of isoflurane on the tryptophan fluorescence anisotropy of myoglobin. Data points are the means, and error bars are the SEM of 25 different measurements. The lines through the data points were generated using linear regression analysis.

Fig. 5. Tryptophan fluorescence anisotropy of BSA (5 μM) as a function of temperature. Data points are the means, and error bars are the SEM of 16 different measurements. The line through the data points was generated using linear regression analysis.
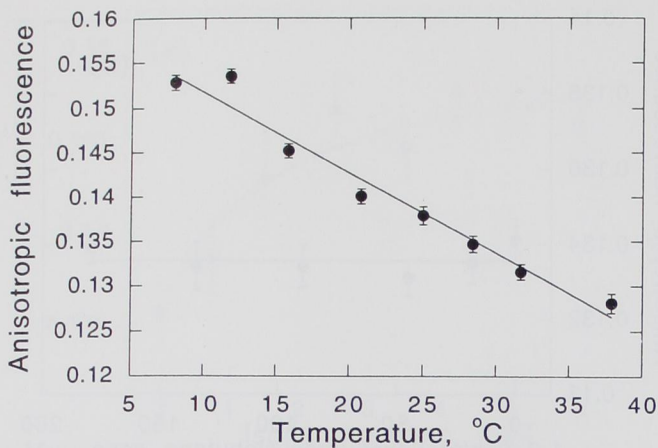
the tryptophan fluorescence anisotropy of BSA was examined and is shown in figure 5. There is a progressive decrease in the fluorescence anisotropy (reflecting an increase in the mobility of the tryptophan residues) as the temperature is increased from 8 to 38°C. The effect of the bound anesthetic agents on indole ring mobility in BSA (fig. 2) is therefore comparable to what results from decreasing the temperature of the protein solution.

Effect of Bound Anesthetic Agents on Protein Stability as Assessed by Near-ultraviolet Circular Dichroism Spectroscopy

Circular dichroism is a form of spectrophotometry that makes use of polarized light and may give rise to positive and negative absorption bands that are conformation dependent. The differential absorption of left and right circularly polarized light after passing through a solution of optically active material (*e.g.*, a protein) results in the transmission of elliptically polarized light. Near-ultraviolet (*i.e.*, 250–330 nm) CD spectra reflect protein tertiary structure[26] and were obtained to examine further the effect of anesthetic agents on the local environment of the aromatic residues in BSA. The motionally constrained tryptophan, tyrosine, and phenylalanine side chains in the native folded form of albumin give rise to the CD spectra shown in figure 6A with broad negative transitions in the 255- to 300-nm range. Adding either halothane or isoflurane to albumin results in only minor changes in these steady-state spectra, suggesting the absence of major structural changes (data not shown). In contrast, elimination of protein tertiary structural con-

tacts by adding 4 M guanidinium chloride leads to the loss of most of the near-ultraviolet CD signal (fig. 6B) as the aromatic side chains are freed to assume additional conformations around the C^{α}-C^{β} and C^{β}-C^{γ} bonds (fig. 1).

Thermal Denaturation of Bovine Serum Albumin as Followed by Near-ultraviolet Circular Dichroism Spectroscopy

Anesthetic binding to BSA has minimal effects on the steady-state near-ultraviolet CD signal arising from the aromatic residues. Changes in the dynamics of the protein are apparent, however, because the bound anesthetic agent increases the tryptophan fluorescence anisotropy. To further investigate the effect of halothane and isoflurane binding on BSA dynamics, thermal denaturation (unfolding) of BSA was performed in the presence of the anesthetic agents.

In the absence of anesthetic agent, T_m for albumin unfolding averaged 61.3 ± 0.2°C (n = 4) for the isoflurane controls and 61.9 ± 0.1°C (n = 4) for the halothane controls. Halothane and isoflurane increase T_m as shown in figure 7. This finding implies that an increase in the overall stability of the native folded protein is observed in the presence of the anesthetic agent, with a shift of the thermal transition to higher temperatures in a concentration-dependent manner (fig. 8).

The increase in T_m was accompanied by an increase in ΔH for unfolding as predicted, because $d(\Delta H)/dT = \Delta C_p$ (the change in the heat capacity) is positive for protein unfolding, because of the exposure of previously buried hydrophobic residues to water.[27] Figure 9 shows the best fit lines for ΔH *versus* T_m, yielding $\Delta H = 157 +$
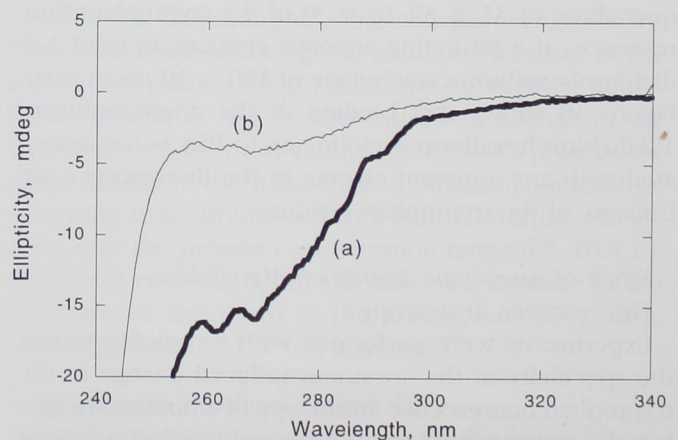

Fig. 6. (A) Near-ultraviolet circular dichroism spectrum of BSA (22 μM) in 130 mM NaCl, 20 mM sodium phosphate, pH 7.0. (B) Decrease in the ellipticity after the addition of 4 M guanidinium chloride.
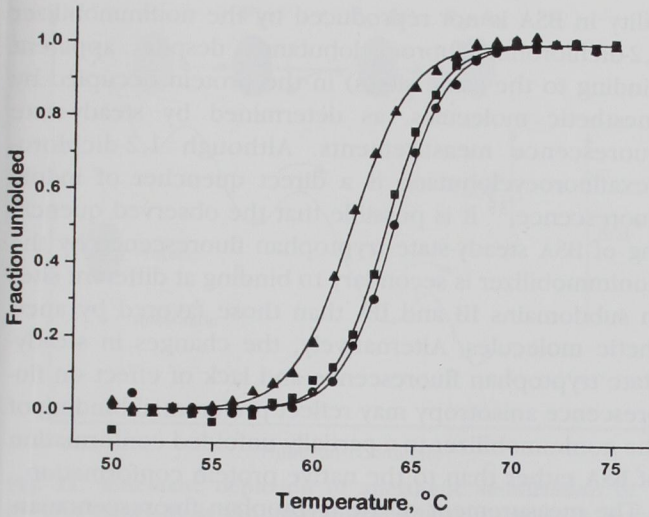
Fig. 7. Fraction of BSA (22 μM) molecules unfolded (f_u) as a function of temperature in 130 mM NaCl, 20 mM sodium phosphate, pH 7.0, followed by the disappearance of the ellipticity at 270 nm. Halothane *(filled circles)* and isoflurane *(filled squares)* shift the denaturation transition to higher temperatures in a concentration-dependent manner. Solid curves represent fits to the data, determined as described in methods.

5.6(T_m − 61.3) for isoflurane and ΔH = 158 + 5.7(T_m − 61.9) for halothane, where ΔH is in kcal/mol and T_m is in °C. These values for ΔH were used in equation 5 to calculate the normalized equilibrium constant K_0/K_{app}, which was plotted against the anesthetic concentration, as shown in figure 10, allowing calculation of dissociation constants for the anesthetics. Best fits to the data in figure 10 yield K_d values of 0.98 ± 0.10 mM for isoflurane and 1.0 ± 0.1 mM for halothane.
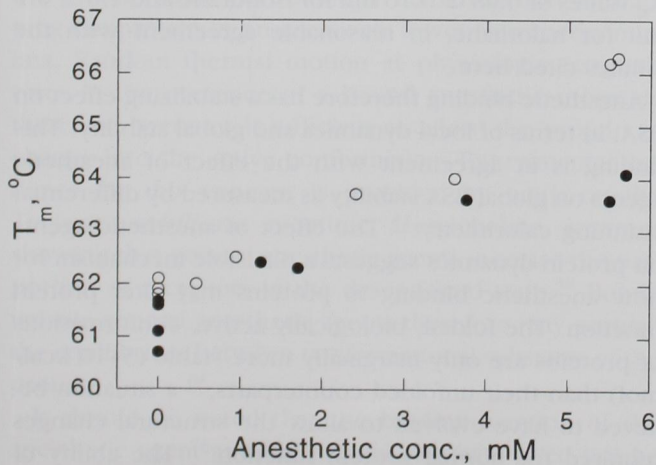

Fig. 8. Variation of the transition midpoint temperature (T_m) for BSA unfolding with the concentration of isoflurane *(filled circles)* and halothane *(open circles)*. Both agents increase the folded stability of the protein in a concentration-dependent manner.
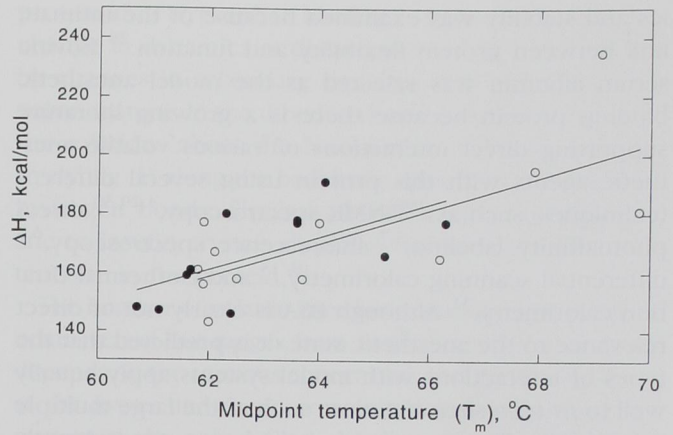

Fig. 9. Variation of the change in the unfolding enthalpy (ΔH, in kcal/mol) with the midpoint transition temperature (T_m, in °C). Each point represents a separate experiment: filled circles for the isoflurane series and open circles for the halothane series. As expected, ΔH increased with increasing T_m. Best fit lines were ΔH = 157 + 5.6(T_m − 61.3) for isoflurane and ΔH = 158 + 5.7(T_m − 61.9) for halothane. These best fit lines for ΔH were used to calculate the normalized equilibrium constants (shown in fig. 10) from the change in T_m attributable to added anesthetic agents.

Discussion

The goal of this study was to gain an understanding of fundamental mechanisms whereby a bound anesthetic molecule on a protein might modify protein function. The effect of bound anesthetic agents on protein dynam-
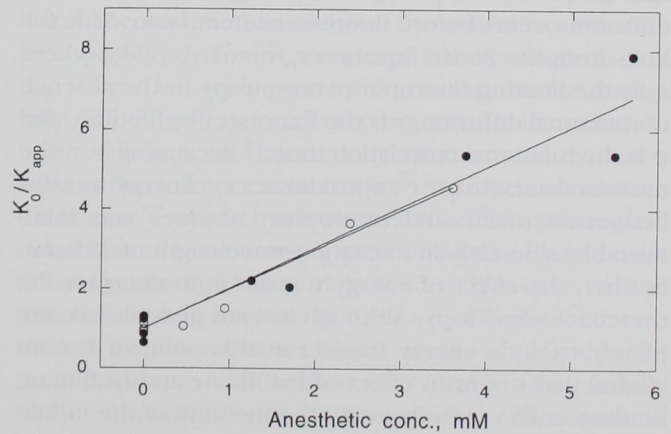
Fig. 10. Plot of the normalized equilibrium constant K_0/K_{app} *versus* anesthetic concentration. K_0 is the equilibrium constant for BSA unfolding in the absence of anesthetic agent, and K_{app} is the equilibrium constant in the presence of the indicated anesthetic concentration. Each point represents a separate thermal unfolding experiment. Open circles are for the halothane series, and closed circles are for the isoflurane series. As described in methods, the slope of this plot ($1/K_d$, equation 6) gives the dissociation constant for the anesthetic agent bound to the native protein molecule.

ics and stability was examined because of the intimate link between protein flexibility and function.[28] Bovine serum albumin was selected as the model anesthetic binding protein because there is a growing literature supporting direct interactions of various volatile anesthetic agents with this protein using several different techniques, such as ^{19}F-NMR spectroscopy,[4,29,30] direct photoaffinity labeling,[5,7] fluorescence spectroscopy,[6,8] differential scanning calorimetry,[15] and isothermal titration calorimetry.[31] Although BSA is clearly not of direct relevance to the anesthetic state, it is predicted that the types of interactions with model systems apply equally well to *in vivo* sites of action, such as the large multiple subunit membrane proteins, which are not currently amenable to detailed biophysical analyses.

Two volatile general anesthetic agents that bind to BSA were found to cause an increase in the fluorescence anisotropy of the tryptophan residues. Fluorescence depolarization of protein tryptophan side chains may result from three distinct processes: (1) Brownian rotational diffusion of the protein as a whole; (2) internal fluctuations of the indole rings relative to the protein backbone; and (3) energy transfer between excited state moieties.[32] The contribution of Brownian rotational diffusion is small in the case of a relatively large protein such as albumin (66 kDa) because the average excited state lifetime of the tryptophan residues of 6.1 ns[33] is short compared with the rotational correlation time of albumin of 42 ns,[34] implying that no significant rotational diffusion occurs before fluorescence emission. This follows from the Perrin Equation $r_o/r = 1 + (\tau/\phi)$, where r_o is the limiting fluorophore anisotropy in the absence of rotational diffusion, τ is the fluorescence lifetime, and ϕ is the rotational correlation time.[14] Because $\phi \gg \tau$, the measured anisotropy r approximates r_o. Energy transfer between tyrosine and tryptophan residues was minimized by selecting an excitation wavelength of 295 nm. Further, the effect of energy transfer is to decrease fluorescence anisotropy. Although it is not possible to completely exclude energy transfer at this point, it is concluded that the main effect of halothane and isoflurane binding to BSA is to decrease the mobility of the indole rings. In the absence of anesthetic agent, the rotations of the indole rings are limited by van der Waals repulsions because of collisions with neighboring residues. The presence of the bound anesthetic agent therefore further increases the likelihood that the indole rings will experience steric inhibition to rotation about the dihedral angles χ^1 and χ^2 (fig. 1).

The anesthetic-induced attenuation of indole ring mo-

bility in BSA is not reproduced by the nonimmobilizer 1,2-dichlorohexafluorocyclobutane, despite apparent binding to the same site(s) in the protein occupied by anesthetic molecules, as determined by steady-state fluorescence measurements. Although 1,2-dichlorohexafluorocyclobutane is a direct quencher of indole fluorescence,[35] it is possible that the observed quenching of BSA steady-state tryptophan fluorescence by the nonimmobilizer is secondary to binding at different sites in subdomains IB and IIA than those favored by anesthetic molecules. Alternatively, the changes in steady-state tryptophan fluorescence and lack of effect on fluorescence anisotropy may reflect preferential binding of the nonimmobilizer to a partially unfolded conformation of BSA rather than to the native protein conformation.

The measurement of the tryptophan fluorescence anisotropy of BSA as a function of added anesthetic agent allowed a quantification of the binding energetics of isoflurane and halothane. Figure 2A shows that isoflurane binds with a K_d of 1.6 ± 0.4 mM. This value is comparable to the K_d values of 1.4 ± 0.2 and 1.3 ± 0.2 mM determined using ^{19}F-NMR spectroscopy[4,30] and the value of 1.5 ± 0.2 mM reported by Eckenhoff and Shuman[5] using photoaffinity labeling. Halothane bound to BSA with a K_d value of 1.3 ± 0.3 mM using fluorescence anisotropy measurements (fig. 2B), again comparable to the value of 1.3 ± 0.2 mM obtained using ^{19}F-NMR spectroscopy,[29] the value of 1.8 ± 0.2 mM determined using intrinsic fluorescence quenching measurements,[6] and the value of $0.3 - 0.5$ mM reported using photoaffinity labeling.[5] The thermal denaturation experiments yielded K_d values of 0.98 ± 0.10 mM for isoflurane and 1.0 ± 0.1 mM for halothane, in reasonable agreement with the studies cited here.

Anesthetic binding therefore has a stabilizing effect on BSA, in terms of local dynamics and global stability. This finding is in agreement with the effect of anesthetic agents on global BSA stability as measured by differential scanning calorimetry.[15] The effect of anesthetic agents on protein dynamics suggests a plausible mechanism for how anesthetic binding to proteins may alter protein function. The folded, biologically active, conformations of proteins are only marginally more stable (5–10 kcal/mol) than their unfolded counterparts,[36] a situation believed to have evolved to allow the structural changes required for normal protein function.[37] The ability of both protein receptors to bind ligands, and for enzymes to transform substrates, is dependent on protein motion.[28] Any alteration in the stability of a given conformational substate is predicted to alter the equilibrium
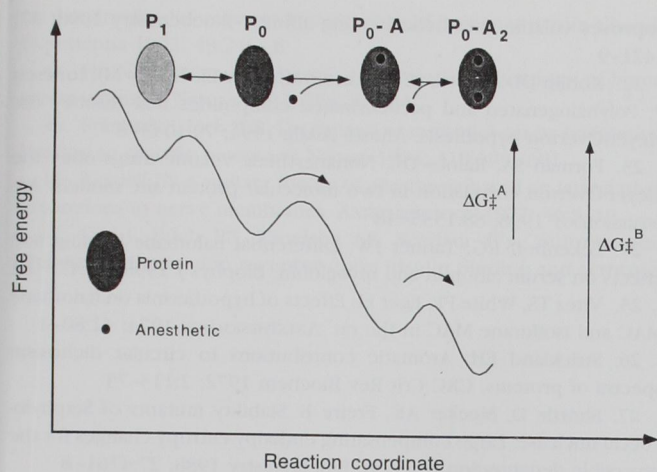
Fig. 11. Schematic depiction of anesthetic stabilization of a protein target. P_0 is the uncomplexed protein target, A is the anesthetic bound to the protein, P_1 is the active form of the protein, and $\Delta G^{\ddagger A}$ and $\Delta G^{\ddagger B}$ are the activation energies required to transform the protein conformation to its active state, from the unliganded condition and after binding of a single anesthetic molecule, respectively. Binding of sequential anesthetic molecules lowers the free energy of the system, thereby increasing the activation energy barrier that must be overcome to allow a conformational change required for protein activity.

between the different conformations, by raising the free energy barrier for subsequent conformational changes required for function. By limiting protein dynamics, volatile general anesthetic agents might alter the binding of native ligands to their receptors and thus disturb the function of central nervous system membrane proteins. This concept is shown schematically in figure 11, in which the binding of successive anesthetic molecules to the protein results in additional stabilizing forces, trapping the protein in progressively lower free energy minima. Random thermal motion at physiologic temperatures in the presence of a bound anesthetic molecule therefore becomes insufficient to allow the protein to change into the active conformation. Experimental evidence for this concept is provided by work on the *Torpedo nobiliana* nicotinic acetylcholine receptor showing that general anesthetic agents increase the proportion of receptors in the desensitized state.[38] Bound volatile general anesthetic agents therefore may perturb the equilibrium between conformational substates of the protein.

It should be noted that the binding location of the anesthetic agent and the natural substrate need not be the same for apparent competition to occur. Binding of the anesthetic agent at one site may alter backbone dynamics at distant sites in the protein, preventing substrate binding, as seen in the case of the *trp* aporepressor

protein after tryptophan binding.[39] In the latter case, this change in dynamics at distant sites is not accompanied by changes in secondary structure and may instead involve changes in the dihedral ϕ and ψ angles (describing the rotations about the N-C^α and C^α-C' bonds, respectively; fig. 1) along the backbone without the breaking of hydrogen bonds. Similarly, binding of 1,2-dichloroethane to insulin crystals displaces sulfate ions from a distant site in the protein.[40]

The number of volatile anesthetic binding sites on BSA is controversial but ranges from two[6-8] to four[4] and clearly depends on the aqueous concentration of agent. Therefore, at the lower anesthetic concentrations examined in the current study, only a single binding site was determined based on the thermal denaturation data. Occupancy of the site(s), although not associated with changes in protein secondary structure,[6,8] presumably causes some local, more subtle, structural or dynamic changes because the binding of other ligands such as thiopental,[41] coumadin,[42] and bilirubin[43] is altered by volatile anesthetic agents. Of these, both coumadin and bilirubin bind to the same IIA subdomain as halothane.[44] This finding suggests that volatile anesthetic binding to proteins may compete with binding of endogenous ligands as previously suggested.[45] As indicated earlier, however, this is not necessarily attributable to competition for occupancy of exactly the same physical site on the protein.

Anesthetic agents have been shown previously to have a fluidizing effect on lipid bilayers.[46] The finding that halothane and isoflurane have the opposite effect on local protein dynamics and global stability suggests that the interaction with protein targets may be fundamentally different. Protein stability is generally increased on ligand binding, as reflected by measures of dynamics such as fluorescence anisotropy,[47] hydrogen–deuterium exchange,[39] and Debye-Waller factors.[48] In this regard, halothane and isoflurane appear to behave like conventional ligands. Further work is needed, however, to confirm this finding because in the case of firefly luciferase there is evidence that anesthetic agents actually destabilize the protein and bind most favorably to a partially unfolded conformation.[16] Nevertheless, anesthetic-induced attenuation of protein dynamics as described in the current study and by others[24] is compatible with the clinical observations that (1) hypothermia decreases anesthetic requirements,[25] and (2) increased pressures, which denature proteins,[49] reverse the effects of anesthetic agents.[50]

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