

Molecular Interactions Between Inhaled Anesthetics and Proteins

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I. Introduction

A. General

Inhaled anesthetics can alter the activity of a wide variety of proteins, but the molecular nature of the interactions underlying the functional effect is poorly understood. In this review, we present and discuss the key evidence for potential molecular interactions with the intent of demonstrating that the many functional effects of inhaled anesthetics are mediated by direct interactions with protein. Although the overall implication is, of course, that these direct interactions ultimately underlie

the behavioral state known as "anesthesia," we emphasize that this is not intended to be a review of the various theories of anesthetic action. We restrict this discussion to the more fundamental question of how inhaled anesthetics interact with proteins to alter their activity. At this point, too little is known about the molecular targets of inhaled anesthetics to relate these fundamental interactions to pharmacology. We will focus on soluble proteins, because the binding and structural investigations in these generally small proteins have been most productive and are not confounded by the presence of lipid, as in the case of membrane proteins. We recognize that soluble proteins may not mediate important components of anesthetic action; there seems to be consensus that membrane proteins serve this role (Pocock and Richards, 1991; Franks and Lieb, 1994; Harris et al., 1995). However, the information derived from the soluble proteins should allow an unambiguous definition of binding character and energetics, and the local and global conformational consequences of anesthetic binding, which almost certainly are relevant to the more

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^b Abbreviations: A, anesthetic; ATPase, adenosine triphosphatase; BSA, bovine serum albumin; CD, circular dichroism; DCE, 1,2-dichloroethane; GABA, gamma-aminobutyric acid; ΔG° , Gibbs free energy change; k_1 , forward rate constant; K_d , dissociation constant; nAChR, nicotinic acetylcholine receptor; P, protein target; PLL, poly(L-lysine); S-H, sulfur-hydrogen.

complex membrane proteins. Because of the large number of compounds that can produce the behavioral state termed "anesthesia," we will further restrict our focus to the inhalational anesthetics, including those with vapor pressures higher than atmospheric at room temperature, such as xenon and nitrous oxide. Although receptor-specific compounds (such as α_2 -adrenergic agonists, N-methyl-D-aspartate antagonists, opiates, and benzodiazepines) can produce behavioral states resembling anesthesia, their much higher affinity and specificity suggest that their molecular interactions are different from those of general anesthetics, which are characterized by low-affinity interactions and widespread effects. Even here, however, there may be overlap in how high- and low-affinity compounds influence the activity of proteins.

B. History

An interaction between inhalational anesthetics and protein was first suggested by Claude Bernard (1875), who stated that diethyl ether and chloroform produced a reversible coagulation of the "albuminoid" cell contents and that this somehow caused anesthesia. Later, Moore and Roaf (1904, 1905) reported that both ether and chloroform were more soluble in serum or hemoglobin solutions than in water or saline, suggesting some sort of attractive (binding) interaction with the protein. Further, they proposed that the uptake of anesthetics by "proteoid" rather than "lipoid" components was responsible for the production of anesthesia. Countering this idea was the independent work of Overton and Meyer, at about the same time, resulting in the famous correlation of anesthetic potency and olive oil solubility, indicating that anesthetics work by interacting with the olive oil-like components of the cell (Lipnick, 1986, 1989; Miller, 1993).

Nevertheless, observations in support of anesthetic interactions with protein continued. In 1915, Harvey reported that *n*-alkanols, diethyl ether, and chloroform reversibly depressed the luminescence of certain marine bacteria and that inhibitory potency correlated with *n*-alkanol chain length. Because the light from these microorganisms derives from the activity of specific enzymes, collectively called "luciferases," these observations suggested that not only could anesthetics bind to protein, but the binding could be associated with an alteration in protein activity. This early work spawned a whole series of more recent investigations with purified light-emitting proteins (see Sections II.B., II.D.1., III.B.) with the goal of demonstrating and characterizing direct anesthetic-protein interactions.

Extending studies on protein activity to protein structure, Östergren (1944) postulated that general anesthetics such as the *n*-alkanols, nitrous oxide, chloroform and trichloroethylene exert their effects on the lipophilic portion of proteins, based on his observations of anesthetic effects on the mitotic spindle. This speculation finally

began to reconcile the observations of Overton and Meyer with those of protein target proponents. The interaction of anesthetics with these lipophilic (hydrophobic) domains was hypothesized to lead to decreased flexibility of the protein (Östergren, 1944). Given the recent appreciation for the importance of marginal stability and conformational dynamics to protein function, this was a remarkably precocious proposal.

Almost thirty years later, the description of the structure of the cell membrane (Singer and Nicolson, 1972), when combined with the observations of Overton and Meyer, once again led the field of anesthetic mechanisms research into the lipid bilayer, which unfortunately has produced much ambiguity. Years of study have indeed verified anesthetic influences on the properties of lipid bilayers (Trudell, 1991; Gruner and Shyamsunder, 1991; Qin et al., 1995; Cantor, 1997), but quantitative problems and heterogeneity of anesthetic effects have forced many investigators to refocus their attention to protein, now with an improved appreciation of the importance of hydrophobic forces to protein structure and function. Further, the functional inseparability of lipid and protein in the cell membrane is now better understood and thus provides a rich environment for reconciliation of the lipid and protein proponents.

II. Anesthetic Binding to Protein

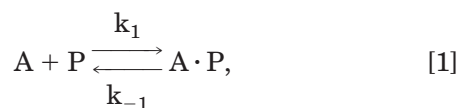
A. Equilibrium Kinetics

For an anesthetic to have a direct effect on protein activity, it must first bind to the protein in some way. This has been a source of confusion, because, traditionally, anesthetics have been considered to partition into, and not necessarily bind to, biological macromolecules. However, the confusion arises from the fact that only weak binding forces are involved. Partitioning generally refers to the distribution of solute between two or more bulk solvents, whereas binding is generally viewed as a molecular event. Nevertheless, the two are clearly related. For example, excess partitioning of a compound into an aqueous solution of protein as compared with water alone is due to binding interactions with the protein. Even partitioning between bulk solvents can be considered to result from preferential binding of the solute to one solvent molecule as compared with the other. Favorable partitioning of halothane into octanol as compared with octane, for example, is probably due to weak electrostatic interactions (see Section II.C.2.) between the more polar octanol molecule and halothane. Several such electrostatic interactions acting in concert, combined with steric effects, produce the higher-affinity binding interactions between more familiar ligands and receptors. Therefore, the distinction between binding and partitioning is in reality more semantic than real.

It may also be necessary to adjust one's view as to the meanings of high and low affinity and specific and non-specific binding in the context of anesthetic ligands with

limited interactive capability. Specific binding is generally considered to be high-affinity and low-capacity (saturable) binding and is usually linked with drug action. Nonspecific binding is characterized as low-affinity and high-capacity binding and implies weak interactions at sites not relevant to a compound's principal pharmacological action. Nonetheless, specific and nonspecific are relative terms; even binding characterized as "nonspecific" for a given drug may be saturable with sufficiently high drug concentration and could produce functional effects distinct from those mediated by specific sites. The primary action of a high-affinity drug, like an opioid or a catecholamine, is in general produced at a drug concentration less than indicated by receptor K_d values, resulting in minimal nonspecific binding. "Anesthesia," on the other hand, occurs at an inhaled anesthetic concentration suspected of producing considerable nonspecific binding. Because inhaled anesthetics produce minimal detectable effects at much lower concentrations, it is likely that what would conventionally be termed "nonspecific binding" is important for the action of inhaled anesthetics. This is especially true in the case of the membrane proteins, given the relatively high concentration of drug in at least part of the protein's solvent (lipid), and the hydrophobicity of these proteins. On the other hand, the discrete, saturable anesthetic binding sites recently found in some soluble proteins, which better merit the term "specific", appear to mediate only modest functional consequences. Therefore, rather than forcing inhaled anesthetics into the familiar ligand-receptor mold, it will be necessary to view the interactions that these clinically important compounds have with protein with an unbiased eye to ultimately understand the underlying mechanisms of their many actions.

The binding of an anesthetic (A) to a protein target (P) can be described as follows:



with forward and reverse rate constants k_1 and k_{-1} , respectively. The dissociation constant of this interaction, K_d , is defined by:

$$K_d = \frac{k_{-1}}{k_1} = \frac{[A][P]}{[AP]} \quad [2]$$

As is apparent from Equation [2], the K_d is expressed in molar units and is important because it reflects the strength and average lifetime of an interaction. The lower the K_d , the stronger the interaction and the longer compound A is immobilized in the binding site of P . In general, a strong interaction increases the probability that there will be a structural, dynamic, and functional consequence of an interaction between A and P . What are the dissociation constants for inhalational anesthetics? Initial estimates are possible. If we assume

that anesthetic action results from binding to a limited number of sites and that occupancy of these sites is linearly coupled to the clinical response, then the aqueous EC_{50} concentration will approximate the K_d , which, for most inhalational anesthetics, is between 200 and 300 μM (Franks and Lieb, 1993). Using this approximate K_d , and assuming that the forward rate constant (k_1) is diffusion-limited (limited only by the time required to have reactants collide through diffusion, which for a protein and small ligand is $\sim 10^9 \text{ M}^{-1} \cdot \text{sec}^{-1}$), we calculate a dissociation rate constant (k_{-1}) of ~ 0.25 to $1.0 \cdot 10^6 \text{ sec}^{-1}$, implying that binding sites are occupied for lifetimes of only 1 to 4 μsec . A comparison with more familiar high-affinity ligands like the catecholamines or the peptide hormones (K_d values of 2 to 10 nM; Cooper et al., 1982), which have site occupancies of many minutes, emphasizes how weak inhaled anesthetic binding interactions really are. However, the improbable assumptions associated with this simplistic analysis make such estimates only tentative. The probability of multiple binding sites and nonlinear coupling between occupancy and response would suggest that anesthetic K_d values are really much higher than observed EC_{50} values. This is, of course, entirely in keeping with the behavior of other ligands, like the opioids. Fentanyl, for example, has analgesic (Olkola et al., 1995) or inhibition of intestinal motility (Magnan et al., 1982) EC_{50} values in the 1 to 10 ng/ml plasma range, translating to 0.5- to 5 nM free concentrations (assuming 85% protein binding). Measured fentanyl μ -opioid receptor dissociation constants, on the other hand, range from 7 to 400 nM (Magnan et al., 1982; Carroll et al., 1988), the higher numbers (lower affinity) generally reflecting more physiological conditions (e.g., the presence of sodium, G proteins, and GTP). Although possibly expected in the highly amplified G protein-coupled receptor response, a wide separation of EC_{50} and K_d is also seen in other receptor systems, like the ligand-gated ion channels. Pancuronium produces a 50% suppression of twitch tension at a free drug concentration of 0.3 to 0.4 μM (Shanks, 1986), whereas the K_d for the nicotinic acetylcholine receptor (nAChR) has been reported to be $\sim 60 \mu\text{M}$ (Loiacono et al., 1993). In addition, whereas a 10 nM free concentration of diazepam is anxiolytic (Greenblatt et al., 1980), the K_d of this ligand for the gamma-aminobutyric acid_A (GABA_A) receptor is estimated to be at least four times that value (DeLorey and Brown, 1992). Therefore, although K_d is often higher than functional EC_{50} , the magnitude is not predictable a priori, suggesting that their match is a poor criterion of the functional relevance of binding sites.

How can dissociation constants be determined more precisely? Because individual rate constants are difficult to measure directly, K_d is usually estimated from equilibrium binding studies with radiolabeled ligands (Weiland and Molinoff, 1981). Even this approach, however, has proven challenging for the inhalational anesthetics

because of the difficulty of separating the free from the bound states with such rapidly dissociating volatile compounds. Recently, however, four methods to estimate the K_d for inhalational anesthetic-protein interactions have been reported. First, simple partitioning between the gas phase and a solution of protein, as compared with buffer alone, can be used to estimate the amount of anesthetic bound by the protein (Dubois and Evers, 1992; Dubois et al., 1993). The separation of specific from nonspecific binding is problematic with this approach, however, but occasionally can be achieved by comparison with partitioning into solutions of denatured protein. This, of course, requires sufficient structural knowledge to ensure that the binding domain is actually removed by the denaturing conditions. It is possible that anesthetics bind preferentially to the denatured state of some proteins, rendering it a very poor model of nonspecific binding. Nevertheless, using low pH to denature bovine serum albumin (BSA) for the estimation of nonspecific binding, isoflurane and halothane have been reported to bind to the native pH 7 form of the protein with K_d values of 1.4 mM and 1.3 mM, respectively (Dubois and Evers, 1992; Dubois et al., 1993). Similar estimates of the halothane K_d for binding to BSA have been obtained with ^{19}F -nuclear magnetic resonance (NMR) (Dubois et al., 1993) and tryptophan fluorescence quenching (Johansson et al., 1995a). A slightly lower estimate of the halothane K_d (0.4 mM) was obtained with direct photoaffinity labeling (Eckenhoff and Shuman, 1993), probably because of selective labeling at sites of highest affinity. Nevertheless, all of these K_d values are well within an order of magnitude and therefore are comparable when considering the overall binding energetics. Consistent with our predictions, all of these estimates for the anesthetic binding to albumin are higher than the clinical EC_{50} values. Although possibly indicating that the albumin binding domains do not accurately reflect the character of the site(s) responsible for anesthetic action, it bears repeated emphasis that EC_{50} and K_d need only be similar if one assumes that anesthesia is produced by interactions at few similar sites and that occupancy is linearly coupled to the clinical effect. These assumptions are untenable, considering the widely varied targets for anesthetics reported in the literature and the rarity of linear coupling in biological systems.

The ^{19}F -NMR measurements have also allowed an estimation of the average lifetime ($1/k_{-1}$) of an anesthetic-albumin complex, which is 200 to 250 μsec for isoflurane, or approximately two orders of magnitude longer than estimated above (see Section II.A.2.) (Dubois and Evers, 1992; Xu et al., 1996). Substituting this dissociation rate in Equation [2] results in very high K_d values, in excess of 10 mM. The problem probably lies in the use of a diffusion-controlled rate for k_1 in Equation [2]. "Diffusion-controlled" implies that all collisions between anesthetic and protein target would result in the bound or

complexed state. However, the actual binding site(s) on the protein surface, or the pathway to an interior binding domain, may only involve a small fraction of the protein surface area, and, therefore, the number of collisional encounters resulting in binding should be smaller than total collisions. Further, normal protein dynamics may limit the duration that the binding site is exposed or available, which will also clearly have an effect on k_1 . So if we now use the experimentally derived K_d and k_{-1} values, k_1 can be estimated to be $3 \cdot 10^6 \text{ M}^{-1}\cdot\text{sec}^{-1}$, or almost three orders of magnitude slower than expected for a diffusion-controlled process. It is of interest that this estimate is comparable with the forward rate constants of 10^4 to $10^7 \text{ M}^{-1}\cdot\text{sec}^{-1}$ observed for other ligand-receptor interactions (Eigen and Hammes, 1963; Gutfreund, 1987; Sklar, 1987). Most importantly, this slower than expected association rate is consistent with a limited number of discrete anesthetic binding sites in soluble proteins, rather than simple nonspecific surface (interfacial) binding (Ueda, 1991). We will now consider the binding event in greater detail.

B. Thermodynamics

Energetics or thermodynamics drives all chemical events, including binding interactions and is therefore a reasonable place to begin our consideration of anesthetic-protein interactions. Dissection of the energetics into enthalpic or entropic components yields insight into the forces and groups responsible for a given interaction. Building on our previous discussion, the magnitude of the dissociation constant (K_d) allows calculation of the Gibbs free energy change (ΔG°) associated with anesthetic binding to protein:

$$\Delta G^\circ = RT \ln K_d, \quad [3]$$

in which R is the gas constant and T is the absolute temperature, under molar standard state at pH 7. The Gibbs free energy change corresponds to the work involved in binding a ligand to a receptor and is negative if binding occurs spontaneously at the prevailing temperature and pressure. The size of the Gibbs free energy difference between the free and bound states determines the stability or strength of the interaction. Table 1 lists the change in free energy associated with given K_d values (anesthetic EC_{50} values used as a rough approximation), and an example of a relevant "anesthetic" compound given for each K_d . Note that the change in ΔG° for different K_d values is fairly modest, especially when compared with the bond energy of a typical covalent interaction ($\sim 100 \text{ kcal/mol}$); a ten-fold change in K_d reflects free energy changes comparable to that produced by a single hydrogen bond ($\sim 1.0\text{--}1.5 \text{ kcal/mol}$; Johansson et al., 1995b).

Dissection of the thermodynamics into entropic (ΔS°) and enthalpic (ΔH°) components can provide clues to the types of interactions associated with binding (Testa et

TABLE 1

Free energy of ligand/protein interactions according to K_d and appropriate examples of anesthetic-like compounds for each range

K_d , M	ΔG° , kcal/mol		Anesthetic	Reference
	25°C	37°C		
1.0	0	0	Methanol	Alifimoff et al. (1989)
0.1	-1.4	-1.4	Ethanol	Alifimoff et al. (1989)
0.01	-2.7	-2.8	Diethylether	Franks and Lieb (1993)
0.001	-4.1	-4.3	Chloroform	Franks and Lieb (1994)
0.0001	-5.5	-5.7	Halothane	Franks and Lieb (1994)
0.00001	-6.8	-7.1	Barbiturates	Franks and Lieb (1994)
0.000001	-8.2	-8.5	Propofol	Franks and Lieb (1994)
0.0000001	-10.9	-11.4	Opiates	Kosterlitz and Waterfield (1975)

al., 1987; Raffa and Porreca, 1989). This can be accomplished by analysis of anesthetic binding at different temperatures using the integrated Van't Hoff equation:

$$\ln K_d = \frac{\Delta H^\circ}{RT} - \frac{\Delta S^\circ}{R}, \quad [4]$$

a combination of Equation [3] and the Gibbs-Helmholtz equation:

$$\Delta G^\circ = \Delta H - T\Delta S^\circ \quad [5]$$

A plot of $\ln K_d$ as a function of T^{-1} permits calculation of ΔH° and ΔS° from the slope ($\Delta H^\circ/R$) and intercept ($\Delta S^\circ/R$), respectively, assuming that the changes in enthalpy and entropy are constant over the temperature range examined. Although the majority of energetic data on ligand binding has been obtained using such Van't Hoff plots, more recently, investigators have used isothermal titration calorimetry, allowing direct measurement of the minute change in temperature of a system when ligand binds to protein (Jakoby et al., 1995; Fisher and Singh, 1995; Ueda and Yamanaka, 1997). It is of interest that almost no such data on anesthetic binding to protein exists.

How can such thermodynamic information be interpreted? Binding of a hydrophobic compound to a protein target would suggest an increase in the order of the system (a negative change in entropy), but the more important entropic event may be the elimination of the many "structured" water molecules surrounding the hydrophobic ligand in aqueous solution (Muller, 1990), a situation termed "hydrophobic hydration." Therefore, the net entropic change associated with binding to a target based strictly on this hydrophobic effect is actually positive (favorable). Even further entropic advantage is gained if the ligand binding site in the protein is normally occupied by water molecules (also likely to be "structured"), which are "released" by ligand binding (such as occurs when dichloroethane binds to insulin; see Section II.D.4.). Binding events based strictly on such hydrophobic events tend to be associated with little or no enthalpic change (no change in heat content) unless there are also some weak electrostatic interactions involved (e.g., van der Waals and/or hydrogen bonding;

see Section II.C.2.). These weak attractive forces between ligand and target will improve the stability of the complex (negative free energy change) and decrease the enthalpy (i.e., decrease the heat content, an exothermic process). Typical ligand-receptor interactions are very stable, being dominated by large enthalpic components; anesthetic-protein interactions are thought to be less stable, with small ΔH° values.

Thermodynamic analyses of anesthetic-protein interactions have, to date, been limited to only a few systems, most of these being the simpler soluble proteins. The Overton-Meyer relationship would emphasize the importance of hydrophobic interactions, suggesting that entropic contributions dominate. However, using a light-emitting enzyme purified from fireflies, Dickinson and colleagues (1993) reported that the interaction between inhalational anesthetics and firefly luciferase was also characterized by a negative enthalpy at 20°C, showing that heat was released when anesthetics bind to this protein and was consistent with some electrostatic attraction between target and anesthetic. This is in accord with earlier work by Ueda and Kamaya (1973), and, as suggested above, implies that anesthetic binding events are more complex than simple hydrophobic partitioning, at least in these soluble proteins.

Other anesthetic/soluble protein interactions that have been examined energetically include those with albumin, myoglobin, and lysozyme. We have used tryptophan fluorescence quenching to examine the temperature dependence of halothane binding to BSA and have found that the apparent K_d decreases as the temperature is reduced, giving the Van't Hoff plot shown in figure 1. This analysis yields a ΔH° of $-1.9 \text{ kcal}\cdot\text{mol}^{-1}$ and a ΔS° of $+6.0 \text{ cal}\cdot\text{mol}^{-1}\cdot\text{K}^{-1}$, quantitatively similar to the values for the halothane-luciferase interaction (Dickinson et al., 1993). Recently, Ueda and Yamanaka (1997) also reported that chloroform binding to BSA is associated with a negative enthalpy change ($\Delta H^\circ = -2.5 \text{ kcal}\cdot\text{mol}^{-1}$). Similarly, the binding of benzene in a hydrophobic cavity of a T4 lysozyme variant is also primarily an enthalpic process (Eriksson et al., 1992a; Morton et al., 1995). Even xenon binding to myoglobin (Ewing and Maestas, 1970) has been shown to be enthalpy-

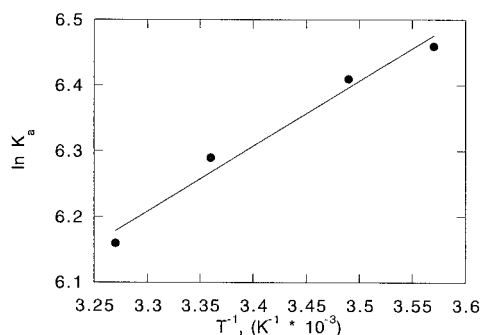


FIG. 1. The dependence of the affinity of BSA ($5 \mu\text{M}$) for halothane at pH 7 in phosphate buffer as a function of temperature as measured by tryptophan fluorescence quenching. Data points are the means of three separate experiments. Buffer pH varies by ± 0.2 units over this temperature range.

driven, with a ΔG° of $-2.9 \text{ kcal mol}^{-1}$ at 25°C . Thus, protein-anesthetic interactions can be characterized by a negative enthalpic change, implying weak electrostatic attractions in addition to the hydrophobic effect, but the relevance of this characteristic to anesthetic action must wait for more studies like these of many different protein systems, including the important membrane proteins.

The energetics of anesthetic binding to membrane-bound protein could be very different, because the target itself is partitioned between two solvents: water and lipid. Few data exist. In the erythrocyte Ca^{2+} -adenosine triphosphatase (ATPase), inhalational anesthetics inhibit activity less (higher IC_{50}) at 25°C as compared with physiological temperature, indicating a more positive ΔH° than found in anesthetic interactions with the soluble proteins mentioned above (Kosk-Kosicka, 1994). This is more consistent with the classical view of the hydrophobic effect and may reflect a greater role of hydrophobicity and a lesser role of electrostatic interactions in anesthetic binding to membrane proteins. Such energetic data could be interpreted as pointing toward the lipid-protein interface, or the lipid bilayer itself, as the relevant functional binding domain in these proteins. Although consistent with studies attempting to localize the inhalational anesthetic binding sites in membrane proteins (see Section II.D.3.) it clearly points toward the need for more work on the thermal dependence of anesthetic effects in membrane systems.

C. Binding Forces

1. *The hydrophobic effect.* Although presented above (See Section II.B.), the hydrophobic effect is a topic of some confusion, so we will discuss it in a little more depth here. The hydrophobic effect is classically thought to result from the attraction of water molecules for each other and the energetic cost of creating a cavity between these strongly hydrogen-bonded molecules to accommodate the hydrophobic molecule (Tanford, 1973; Ben-Naim, 1980). Although this energetic cost can be over-

come by the strong electrostatic attraction between charged species (like ions) and the relatively high partial charges of water, the weakly polar anesthetic molecules have only feeble interactions with water and are therefore forced or squeezed into macromolecular domains of low hydration when available (e.g., hydrophobic regions), returning the more structured water lining the cavity to its more disordered state. This movement of the anesthetic from bulk water into a hydrophobic domain is therefore a favorable entropic event, consistent with a multitude of partitioning studies between organic solvents and water at 25°C (Tanford, 1973; Ben-Naim, 1980; Abraham et al., 1990).

Despite the suggestion of weak electrostatic attractions between anesthetic and protein (negative enthalpy), the Overton-Meyer relationship indicates that the hydrophobic effect is an important interaction between anesthetic molecules and at least some relevant targets. Indeed, initial attempts to characterize structural features of anesthetic binding sites in soluble proteins (see Sections II.D.2–II.D.4.) have revealed internal cavities lined by hydrophobic amino acids. This preference for hydrophobic protein domains was unambiguously demonstrated by the creation of saturable binding domains in poly-(L-lysine) (PLL) by raising the pH above the side group pK_a , converting the random coil polymer to aggregates of hydrophobic α -helices (Johansson and Eckenhoff, 1996; fig. 2).

The energetic cost of creating a cavity within a protein to bind a hydrophobic ligand would be large, so pre-existing space is a probable requirement for binding (Buckle et al., 1996). Although crystallographic and NMR data indicate that native protein side chains fit together with a very high packing density, defects are found in the

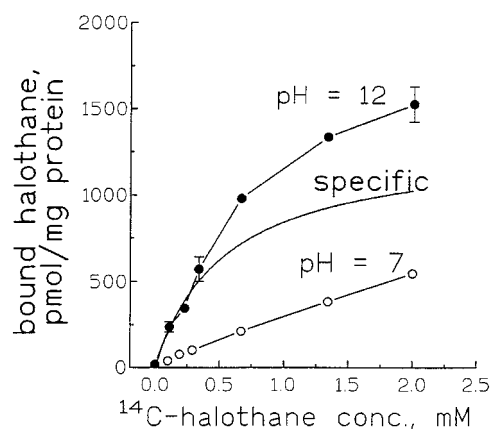


FIG. 2. Direct photoaffinity labeling of PLL (~ 200 residues) at pH 7 and 12. CD spectra (not shown) demonstrated only random coil character at pH 7, and predominantly α -helix at pH 12. Halothane labeling of PLL is substantially increased under alkaline conditions and acquires a saturable character as shown from the curve fitted to the specific binding data (pH 12–pH 7), implying the creation of a suitable hydrophobic domain for halothane immobilization (binding). Reprinted with permission from Eckenhoff and Shuman (1993). conc., concentration.

majority of proteins whose structures have been determined at high resolution. Such packing defects, or cavities, are present in monomeric proteins (Rashin et al., 1986; Hubbard et al., 1994), occur at subunit interfaces of oligomeric proteins (Hubbard and Argos, 1994) and at lipid-protein interfaces of membrane proteins (Lemmon and Engelman, 1994), and may or may not normally be occupied by water molecules (Ernst et al., 1995), depending on size. Richards and Lim (1994) have postulated that although the presence of cavities is energetically costly (probably because of the loss of van der Waals contacts), they have evolved to provide the conformational flexibility required for protein function. This was recently demonstrated in the small protein λ repressor, where site-directed mutations produced an increase in packing density and thermodynamic stability, but a decrease in function (Lim et al., 1994). Internal cavities are therefore an attractive general target for hydrophobic ligands like the inhaled anesthetics.

Cavity volume and shape in natural proteins can vary substantially (Rashin et al., 1986; Hubbard et al., 1994; Hubbard and Argos, 1994; Bianchet et al., 1996; Tegoni et al., 1996), in some cases being large enough to accommodate even bulky general anesthetic molecules like methoxyflurane (2,2-dichloro-11-difluoroethyl methyl ether; 0.144 nm^3). For instance, myoglobin and hemoglobin (see Section II.D.4.) have cavities with volumes of 0.03 to 0.18 nm^3 (Rashin et al., 1986; Tilton et al., 1988), which can accommodate anesthetic molecules sized up to that of cyclopropane or dichloromethane (Nunes and Schoenborn, 1973). However, the shape and preexisting occupancy of these cavities must also influence the binding of anesthetic molecules, because saturable halothane binding to hemoglobin or myoglobin in the low-millimolar concentration range cannot be demonstrated with either photoaffinity labeling (Eckenhoff, unpublished observations) or tryptophan fluorescence quenching (Johansson et al., 1995a) despite adequate cavity volumes. Also consistent with the lack of binding are the minimal effects that halothane has on myoglobin and hemoglobin structure or function (Weiskopf et al., 1971), whereas the smaller dichloromethane clearly binds and produces an allosteric anti-sickling effect on hemoglobin (Schoenborn, 1976). Such cavity volume or shape limitations may be responsible for the well-known "cut-off" effect (Ferguson, 1939; Curatola et al., 1991), whereby anesthetic potency is abruptly lost as the chain length of either *n*-alkanes or *n*-alkanols is increased beyond a specific point.

In summary, the hydrophobic effect is clearly a dominant force in anesthetic binding to protein, and suitable hydrophobic cavities exist in both soluble and membrane proteins, the sterics of which may explain the subtle selectivity for different anesthetic molecules.

2. *Electrostatic interactions.* The original Overton-Meyer correlation of anesthetic potency with lipid solubility was based on olive oil, an inconsistent mixture of

various lipids (Hazzard et al., 1985; Halsey, 1992). A somewhat better correlation has been found for *n*-octanol than for strictly aliphatic solvents (Franks and Lieb, 1978), implying the importance of a polar component at the anesthetic site of action. A further improvement of the correlation is achieved with lecithin, strengthening the hypothesis that the anesthetic site(s) of action have polar and amphipathic features that interact with anesthetic molecules (Taheri et al., 1991; Halsey, 1992). Despite the fact that inhaled anesthetics are themselves formally uncharged, such interactions are electrostatic in nature. Weak electrostatic interactions can be conveniently (but not distinctly) divided into hydrogen bonding and van der Waals (or dispersion) interactions. We will also consider stereoselectivity here, because the spatial distribution of electrostatic interactions within a cavity is likely to be the basis for differential binding selectivity for enantiomers.

a. **HYDROGEN BONDING.** Hydrogen bonds have a central role in protein structure, stability, and function (Fersht et al., 1985; Shirley et al., 1992) and of course are responsible for the structure and properties of water. This relatively strong noncovalent bond arises when two electronegative atoms share (usually asymmetrically) the same hydrogen atom. The atom closest to the hydrogen is often referred to as the "donor" and the other, the hydrogen "acceptor." The electronegative atoms are usually oxygen or nitrogen in biological macromolecules, but sulfur and carbon atoms may also serve as hydrogen bond donors (Burley and Petsko, 1988; Viguera and Serrano, 1995). Sulfur atoms (Morton and Matthews, 1995), the π -electrons of aromatic rings (Levitt and Perutz, 1988) and possibly the electronegative halogens, may serve as hydrogen bond acceptors under some conditions (Murray-Rust et al., 1983; Ramasubbu et al., 1986; Verschuere et al., 1993). Because of the extensive halogenation of most of the inhaled anesthetics, the possibility of halogens participating in a hydrogen bond as a hydrogen acceptor could, in part, explain the improved correlation of the potency with solubility in polar solvents. Figure 3 summarizes some of the possible hydrogen-bonding interactions in which inhaled anesthetics may participate. Experimental evidence supporting the ability of inhalational anesthetics to form hydrogen bonds is largely derived from infrared spectroscopic studies. DiPaolo and Sandorfy (1974a,b), for instance, found that halothane decreased the association between well hydrogen-bonded solutions such as *N*-methylpivalamide and 2,6-diisopropylphenol, indicating competitive hydrogen bonding. Although perfluorinated molecules did not influence these systems, the presence of heavier halogens like chlorine, bromine, and iodine had a progressively greater hydrogen bond weakening effect. This is consistent with the positive correlation between anesthetic potency and halogen size (Lipnick, 1986; Targ et al., 1989). The importance of the halogens is further emphasized by studies with the bacterial enzyme,

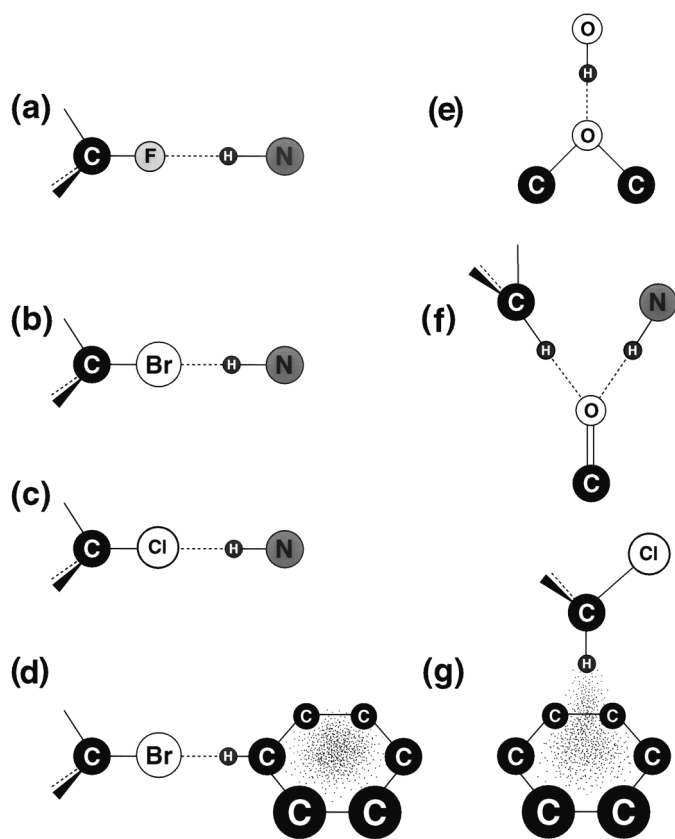


FIG. 3. Potential electrostatic interactions for anesthetics. Panels (a) through (d) represent hydrogen bonding between anesthetic halogens and amid hydrogens, or peripheral aromatic ring hydrogens. Panel (e) shows an ether oxygen (e.g., from isoflurane or desflurane) acting as a hydrogen acceptor for a hydroxyl hydrogen from a serine residue, for example. Panel (f) represents an intrapeptide bond between a carbonyl oxygen and an amide hydrogen, with an anesthetic acting as a hydrogen donor, the implication being that the anesthetic may interact favorably with this group through hydrogen bonding without disruption of the native hydrogen bond. Panel (g) suggests an electrostatic interaction between an "acidic" hydrogen on an anesthetic and the π -electrons of the aromatic ring.

haloalkane dehalogenase. Both of the chlorine atoms of 1,2-dichloroethane have been crystallographically shown to interact with the indole NH groups (as in fig. 3a-c) of two tryptophan residues and the aromatic hydrogens of two phenylalanine residues (as in fig. 3d) in the internal active site of this bacterial enzyme (Verschuere et al., 1993; see Section II.D.4. and fig. 4). The chlorine-nitrogen, or chlorine-carbon bond lengths, however, are longer than the typical hydrogen bonds of water, suggesting a relatively weak interaction and are consistent with the high K_m (~ 1 mM) for dichloroethane dehalogenation by this enzyme.

Inhaled anesthetics could also serve as hydrogen donors in a binding interaction (fig. 3f,g). The few hydrogens on halogenated anesthetics are relatively acidic in character, meaning they possess a partial positive charge because of the electron-withdrawing effect of the halogens, especially fluorine. Hydrogen bond donor ability is closely related to the magnitude of this partial

charge. Accordingly, it has been found that anesthetics with a single or few hydrogen atoms in addition to the halogens are more potent than the perhalogenated analogs (Hansch et al., 1975; Eger et al., 1994). Also, there is a reasonably good correlation between anesthetic potency in tadpoles (Brockerhoff et al., 1986), and possibly for firefly luciferase inhibition (Abraham et al., 1991), with the relative ability to donate such protons in hydrogen bonds. The ability of anesthetics like halothane to donate a hydrogen bond, however, is questioned by the modest partial charge (+0.13 e) on the sole hydrogen (Scharf and Laasonen, 1996), only slightly greater than that on an aliphatic hydrogen (+0.10 e; Burley and Petsko, 1988), and considerably less than that on a water hydrogen (+0.30 e; Williams and Jan, 1987). Although this does not rule out the participation of halothane's hydrogen in binding interactions, such weak hydrogen-bonding capacity does render unlikely the concept that these compounds successfully compete for native hydrogen bonds (those responsible for secondary structure) in proteins.

b. VAN DER WAALS INTERACTIONS. van der Waals forces are ultimately derived from Coulomb's law of attraction between unlike charges. Given the structure and the lack of formal charge on the inhalational anesthetics, it is clear that van der Waals forces must play an important role in the stabilizing energetics that govern binding to a protein. The attractive van der Waals forces include dipole-dipole, induced dipole-dipole, and London interactions. Dipole-dipole is the easiest to visualize and results from attraction of unlike partial charges on molecules and groups. This van der Waals force is likely to be relevant for inhalational anesthetic binding because most of these molecules have a permanent dipole moment. Induced dipole-dipole interactions result from the distortion of an atom's electron cloud (polarization) in the presence of a strong dipole moment (such as in a protein cavity lined by polar residues). Polarizability is a function of atomic mass and thus occurs more prominently with the larger halogens (chlorine, bromine) included on some of the inhalational anesthetics. Accordingly, it is interesting to note that, for a given structure, both anesthetic potency and degree of metabolism are progressively increased as heavier halogens are substituted (Targ et al., 1989; Harris et al., 1992b), suggesting that this type of van der Waals force is important in producing binding interactions in some relevant target. London interactions are a combination of the two. Normal fluctuations in the charge distribution of the electron clouds of one atom influences that of one nearby, resulting in attraction because of momentarily opposed dipole moments. The energy of van der Waals interactions varies as a function of r^{-6} , rendering these forces significant only at very close range. However, once proximity reaches the sum of their van der Waals radii, a strong repulsive force replaces the attraction between two atoms (Gray, 1994), an important consideration for

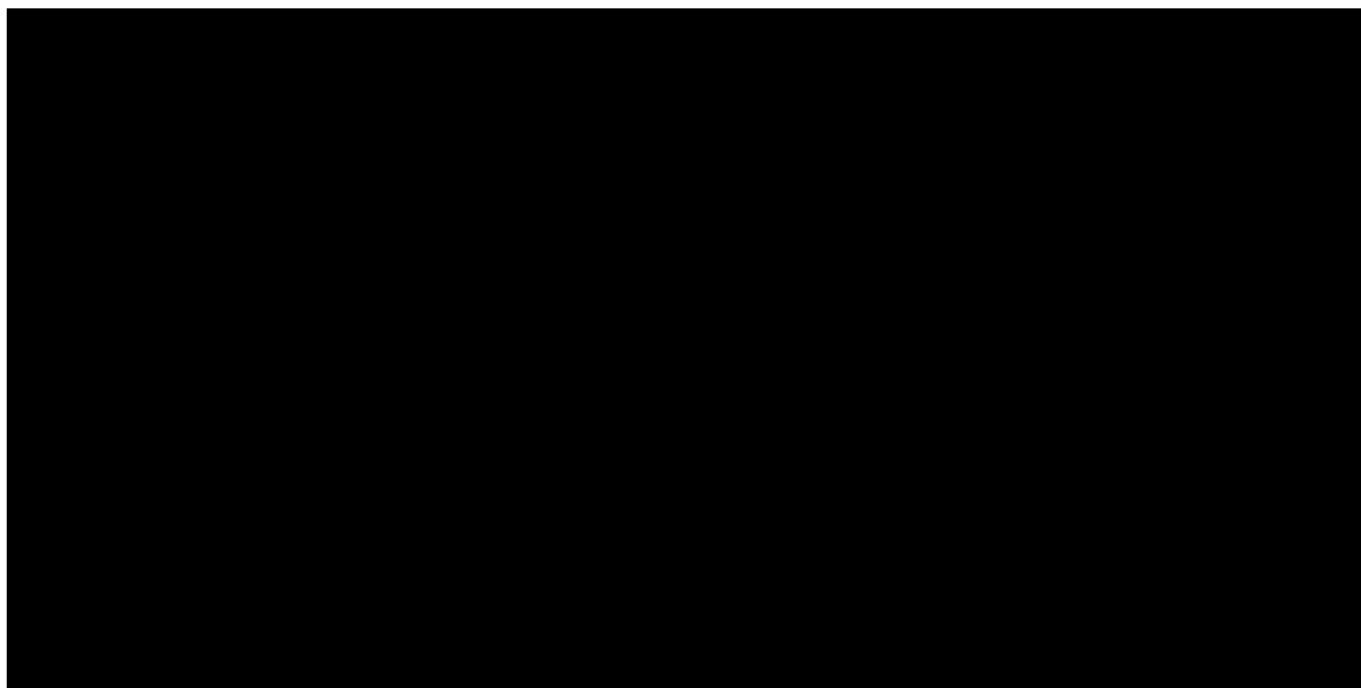


FIG. 4. Binding of the substrate 1,2-dichloroethane to the active site of dehaloalkane dehalogenase from *X. autotrophicus*. (a) One of the chlorine atoms electrostatically interacts with the ring nitrogen protons of Trp 125 and 175, whereas the other chlorine interacts with the side chains (probably peripheral ring hydrogens) of Phe 128 and 172. (b) Crystallographic coordinates for this enzyme at 4°C and in the presence of 1,2-dichloroethane (DCE) were obtained from the Protein Data Bank, and RasMol v2.5 (Glaxo Research and Development, Greenford, Middlesex, UK) was used to produce this image. DCE and the interacting aromatics shown in panel a are shown here in spacefilling, and the remainder of the backbone and side chains shown in wireframe. DCE carbons are gray, and chlorines are green. The two phenylalanines are shown in yellow, with the closest carbon shown in dark blue. The tryptophans are orange, with the nitrogens shown in red. Note the tight fit of this ligand in this internal binding pocket and the close apposition of the two chlorines to the four aromatic side chains. It is of interest that this presumably optimized binding site for this ligand/substrate has an association constant of only 1250 M^{-1} . Adapted from Verschueren et al. (1993).

steric constraints to binding interactions. Xenon binding to myoglobin is an example of binding that must occur entirely through van der Waals interactions, being limited to induced dipole-dipole and London-type interactions. The permanent dipole moments of the clinically used inhaled anesthetics allow all three types of van der Waals forces to act in the stabilization of binding interactions.

In summary, the evidence pointing toward an electrostatic component in inhaled anesthetic binding interactions with protein may be explained by any, or all, of the weak forces discussed in this section. Although it is possible that a single force may dominate some binding interactions (xenon, for example), it seems more likely that all are called into play with the varied interactions of inhaled anesthetics with their multiple targets. Far more work will be required to characterize which forces (if any) control the binding interactions with functionally relevant targets.

c. **STEREOSELECTIVITY.** One of the determinants of ligand binding to a protein target has to do with the spatial arrangements of the important interactive groups in the binding cavity or pocket. When combined with the restraints of cavity shape and size, these spatial arrangements may produce chirality of ligand bind-

ing and thereby stereoselective function. Although the terms are often used interchangeably, stereospecific means that only one optical isomer has activity, whereas stereoselective implies isomers with differing potencies. In the latter case, both (or more) isomers bind, but with differing affinities and, perhaps, consequences. The small-size, low-affinity and small electrostatic interactive potential of the inhaled anesthetics suggest that the term “stereoselective” should apply and that the potency difference between enantiomers should be small. Indeed, Pfeiffer’s (1956) rule suggests that the inhaled anesthetics should demonstrate almost no stereoselectivity, based on the average required dose (mass) of a drug. Accordingly, three in vivo studies have found the (+) isomer of isoflurane to be less than twice as potent as the (–) isomer (Harris et al., 1992a; Lysko et al., 1994), with the most recent rat study showing a statistically insignificant 17% difference (Eger et al., 1997). In vitro, several studies have also shown a small difference using various models (Franks and Lieb, 1991; Eckenhoff and Shuman, 1993; Quinlan et al., 1995). The enantiomers of the even simpler inhaled anesthetic, halothane, had slightly different immobilizing potency in specific mutants of the nematode *C. elegans* (Sedensky et al., 1994), and a 2- to 3-fold difference in metabolism in vivo as

determined by quantitation of trifluoroacetylated liver proteins (Martin et al., 1995). Differences in the potency of halothane in mammals has yet to be reported. The results strongly suggest that there is a small degree of chirality in some functionally relevant sites, clearly pointing toward proteins as opposed to lipid targets. However, there are at least three important caveats of these observations. First, modest stereoselectivity in the action of inhalational anesthetics has now been observed in many reductionist systems, limiting the practicality of this criterion in searching for a single important site of action. Second, even the less potent isomer produces anesthesia, suggesting that distinction at the molecular level will be difficult, unless again, a single site of action is proposed; and third, because stereoselectivity suggests spatial constraints to the binding site, it is very unlikely that the many diverse structures producing anesthesia could all satisfy these constraints, again implying multiple sites of action. Although the initial purpose of these studies was to unambiguously demonstrate that interactions with protein (as opposed to lipid) were responsible for anesthetic action, the result has been somewhat less than definitive because of the possibility of lipid-based chirality. To address this, Franks and colleagues (Franks and Lieb, 1991; Dickinson et al., 1994) reported that the isoflurane stereoisomers partition equally into model lipid bilayers, and lower phase transition temperatures equally. Although it remains plausible that other lipid components of biological membranes not yet examined could contribute to stereoselective anesthetic partitioning or function, the data generally indicate multiple protein anesthetic targets.

D. Location and Character of Anesthetic Protein Binding Sites

We have primarily discussed the forces governing the binding interaction, which, as we have seen, begin to define the character of the binding sites themselves. We have predicted, for example, that the binding sites are hydrophobic with some polar character and whose features are sufficiently general to be widespread. These predictions can be tested and our concepts of the forces involved refined through the actual determination and characterization of anesthetic binding sites in protein. Further, and perhaps most importantly, localization of protein binding sites may provide clues for the structural/dynamic consequences of binding. Such location and structural information can be provided by a number of different approaches, from kinetic studies to X-ray crystallography. We have organized this section by experimental approach, because it is a convenient way to cover the available data, and it is important to realize the individual strengths and limitations of each method for characterizing binding sites.

1. *Functional and equilibrium binding assays.* The most common approach found in the literature is to examine whether the anesthetic binding site overlaps

with a native substrate or ligand site. This information may be provided by determining the concentration dependence of the anesthetic effect on some functional assay or an equilibrium (radioligand) binding assay for a previously established ligand. Double reciprocal plots allow the interaction to be classified as competitive, noncompetitive, or some combination of the two. Competitive kinetics are usually interpreted as anesthetic binding at the same site or at an overlapping site in the protein as the ligand. There are numerous examples of this approach, ranging from soluble proteins, such as adenylate kinase (Sachsenheimer et al., 1977), firefly and bacterial luciferases (Adey et al., 1975; Franks and Lieb, 1984), to membrane bound proteins, such as the ligand-gated ion channels or catecholamine transporters (El-Maghrabi and Eckenhoff, 1993; Moody et al., 1993). In firefly luciferase, for instance, the double-reciprocal plot indicates competition with the native substrate luciferin and further suggests that two halothane molecules fit in the luciferin binding site on the protein (Franks and Lieb, 1984), consistent with the approximate molecular volumes of these two ligands.

On the other hand, halothane's effect on serotonin transport and cocaine binding, also shown to be competitive by functional assays, was subsequently shown by other methods to represent a distant effect, termed "allosteric competition" (Martin et al., 1990a,b; Eckenhoff and Fagan, 1994). In functional studies of alkanol interactions with the nAChR, it was not possible to distinguish between overlapping and allosteric sites, but careful analysis suggested direct action on the protein as opposed to the lipid (Wood et al., 1995). Finally, the apparent competition of fatty acids and halothane for serum albumin binding has now been shown to represent structural/dynamic communication between distant sites, because the halothane binding sites (in domain IIA) are distinct from the high-affinity, long-chain fatty acid binding sites on this protein (Carter and Ho, 1994). These examples of competitive allosterism (Miles et al., 1962) suggest that the sole use of functional approaches could lead to error and dictate that complementary approaches be used to confirm the actual location or character of the anesthetic binding site(s). Further discussion of this approach is found in Section II.D.5.

2. *Spectroscopic approaches.* A powerful series of methods for determining the location of anesthetic binding sites, and one to which we will return when considering alterations in protein structure, is spectroscopy of specific reporter groups on the protein target or the anesthetic itself. These reporter groups typically provide information on the character of their immediate environment, which is expected to change with the addition of an anesthetic molecule. However, it bears emphasis that anesthetic-induced changes in this environment might also result from structural or electronic transitions mediated through occupancy of distant sites. As with the functional assay, other methods are necessary

to confirm the location of the anesthetic interaction or to verify the absence of more global structural/electronic effects in the protein.

a. ¹⁹F-NUCLEAR MAGNETIC RESONANCE SPECTROSCOPY. The volatile general anesthetics that are currently in clinical use (e.g., halothane, isoflurane, and desflurane) are heavily fluorinated to decrease metabolism and flammability. Because fluorine is present in biological systems in negligible amounts, the naturally occurring ¹⁹F isotope on the anesthetic can be studied with NMR spectroscopy with little background noise (Wyrwicz, 1991; Danielson and Falke, 1996). We have already discussed the use of this approach to define kinetics, but it can also be used to describe the general features of the anesthetic environment in various biological systems. For example, Trudell and Hubbell (1976) used ¹⁹F-NMR to show that halothane rapidly equilibrated between water and the entire thickness of model bilayers composed of egg phosphatidylcholine. Wyrwicz and colleagues (1983a) found multiple environments for halothane in rabbit sciatic nerve but only a single environment in intact rabbit brain (Wyrwicz et al., 1983b). Of interest is the fact that the single anesthetic environment in brain was characterized as being hydrophobic with some polar component, based on the fluorine chemical shift. The most recent example of the ¹⁹F-NMR reporter approach examined the distribution of sevoflurane in the rat head in vivo, exploiting the presence of three identical trifluoromethyl groups on the anesthetic for signal-to-noise purposes. These authors concluded that there were two distinct environments for this anesthetic and that the more "confined" environment correlated better with the kinetics of behavioral anesthesia than the less confined environment (Xu et al., 1995). Xenon may also be useful as an NMR probe, in that its chemical shift appears to be quite sensitive to its environment (Miller et al., 1981; Xu and Tang, 1997), and as stated in Section II.C.2.b., is a weak inhalational anesthetic. The nature of any of these NMR environments remains unclear and may be difficult to characterize further with this single approach, primarily because the binding kinetics are significantly faster than the relaxation process leading to the NMR signal.

b. PROTON NUCLEAR MAGNETIC RESONANCE SPECTROSCOPY. This technique examines the environment of hydrogen atoms and has been applied successfully to biological macromolecules. However, because of the large number of hydrogen atoms in proteins, one-dimensional NMR spectra are characterized by fairly broad resonances because of overlap of the signal from the many chemically identical protons in slightly different environments. Nevertheless, one-dimensional proton NMR spectroscopy has been used to examine the effect of halothane and methoxyflurane on the structure of human hemoglobin (Barker et al., 1975). Clinical concentrations of these anesthetics caused reversible changes in two peaks in the portion of the spectrum that reported

on the environment of aromatic residues. This was interpreted to be a result of localized pK_a changes brought on by alterations of the dielectric constant in the vicinity of these residues, probably because of subtle but widespread conformational changes. Further work confirmed this result and also reported that perturbations in the aliphatic regions of the spectrum occurred following the addition of halothane (Brown et al., 1976).

More recently, two-dimensional (and now three- and four-dimensional) proton, and heteronuclear NMR spectroscopy (Clare and Gronenborn, 1991; Ernst, 1994; Wuthrich, 1995; Jardetzky, 1996) have been used to solve the structure of smaller (15–25 kDa) soluble proteins. Although these approaches have not yet been applied to the question of anesthetic-protein interactions, they are anticipated to provide much useful information as to the location and character of anesthetic binding domains and possibly the conformational consequences of anesthetic binding. As compared with ¹⁹F-NMR, the slow time scale of proton NMR signal generation may be less of a problem when focusing on protein nuclei.

c. FLUORESCENCE SPECTROSCOPY. Most proteins contain fluorescent aromatic residues, such as phenylalanine, tyrosine, and tryptophan. Of these, tryptophan has the highest quantum yield but is the least prevalent. Such features are advantageous in that the dominant protein fluorescence signal results from a few amino acids, which may have known positions in the protein. For instance, the ~600 amino acid protein, BSA, contains only two tryptophan residues, one of which is in a well-characterized binding cavity (IIA) for small charged aromatic molecules, such as warfarin or triiodobenzoic acid (He and Carter, 1992). Binding an anesthetic in this site, provided it contains a heavy halogen like bromine or chlorine, will quench the fluorescence signal arising from these residues. Therefore, fluorescence measurements can serve as markers for the location of the anesthetic in the protein matrix, because heavy atom (halogen) quenching is thought to occur at only short range (~0.5 nm). The analysis can be extended to examine the thermodynamics and kinetics of anesthetic binding as mentioned above (see Section II.A.) and has the advantage over NMR in that signal generation occurs on a much shorter time scale (10⁻¹² seconds). Also, as mentioned above (see Section II.D.1.), it should be realized that alterations in the structure of a protein by binding of the anesthetic at a remote site could change the environment of a tryptophan residue sufficiently to produce, for example, charge transfer interactions with neighboring residues that quench the fluorescence signal. Such local alterations in the nAChR conformation may be responsible for the recently reported anesthetic-induced changes in the kinetics of the fluorescence enhancement following the binding of an acetylcholine analog (Raines et al., 1995). Thus, to conclude that binding occurs in the vicinity of the fluorescent group, it is necessary to have some independent means of confirming

the location, or of ruling out significant structural changes on anesthetic binding. In the case of albumin, photoaffinity labeling (Eckenhoff, 1996a) confirmed that halothane was binding in the immediate vicinity of tryptophan 134 and 214, and circular dichroism (CD) spectroscopy failed to detect a change in albumin secondary structure with high concentrations (12 mM) of halothane (Johansson et al., 1995a). The two tryptophan residues in another soluble protein, myoglobin, apparently exist in a smaller cavity, or one lacking access, because halothane failed to quench the fluorescence in any reasonable concentrations and, as stated previously, was also unable to bind saturably as determined by photoaffinity labeling.

The effect of anesthetic complexation on individual fluorophore environments can be probed with fluorescence lifetimes analysis (Lakowicz, 1983; Beechem and Brand, 1985; Eftink, 1991) of fluorescence anisotropy measurements (Jameson and Sawyer, 1995) as our understanding of the location of anesthetic binding sites on proteins is further refined. Fluorescence lifetime analysis allows differentiation between static and dynamic quenching mechanisms (Eftink and Ghiron, 1981), yielding insight into the fluorophore-anesthetic interaction. A static quenching mechanism, for example, follows an interaction between the fluorophore and quencher as would be expected between a receptor and ligand; in other words, it follows a binding interaction. This mechanism is characterized by little change in fluorophore lifetime and has been identified as the mechanism underlying the quenching of protein tryptophan fluorescence by halothane (Johansson et al., 1996) or chloroform binding (Johansson, 1997). On the other hand, dynamic quenching is characterized by lifetimes that decrease in proportion to the changes in steady-state fluorescence intensity, indicating a random collisional, and therefore less specific, interaction.

The considerable mobility of tryptophan residue side chains in proteins around the C α -C β bond can be quantified using anisotropic measurements; a decrease in anisotropy reflects an increase in probe mobility (the free, unbound fluorophore would have no anisotropy), whereas an increase in anisotropy reflects restricted mobility of the fluorophore. Because anesthetic-protein interactions may have both local and global dynamic consequences, this approach may be a useful means of measurement. Accordingly, Vanderkooi and colleagues (1977) showed that general anesthetics decrease the fluorescence anisotropy of the hydrophobic probe 1-phenyl-6-phenylhexatriene in phospholipid vesicles, suggesting an increase in membrane fluidity. Similarly, halothane has been shown to increase the rotational diffusion of skeletal muscle sarcoplasmic reticulum Ca²⁺-ATPase labeled with a phosphorescent probe (erythrosin-5-isothiocyanate), also using anisotropy measurements (Karon and Thomas, 1993). Interestingly, halothane had the opposite effect on the cardiac muscle isoform of this

ATPase (Karon et al., 1995), and in both cases, the anisotropic measurements correlated with the effect of halothane on enzymatic activity. Therefore, changes in the fluorescence or phosphorescence anisotropy may provide information on local or global changes in protein structure or dynamics and perhaps suggest how anesthetic binding alters protein function.

Fluorescence approaches will be less definitive in proteins containing many tryptophan residues, because all will contribute to the steady-state fluorescence signal; determination of those whose fluorescence is altered by anesthetic binding would be problematic. Some information, however, could be obtained if the multiple tryptophans were in similar domains, such as the transmembrane sequences of membrane proteins. Also, if an independent means of showing which tryptophans were affected by anesthetics (directly or allosterically) were available, then fluorescence could be used to monitor dynamics.

d. ELECTRON SPIN RESONANCE SPECTROSCOPY. Electron spin resonance or electron paramagnetic resonance is a type of absorption spectroscopy that uses microwave radiation to induce transitions between spin states of unpaired electrons (infrequent in biological macromolecules). These transitions are sensitive to the local environment. Unpaired electrons are found on highly reactive and transient free radicals and, in addition, on the more stable nitroxide spin labels. Therefore, covalent incorporation of nitroxide spin labels into macromolecules permits monitoring of the viscosity, topology, and dielectric features of the spin label environment (Likhtenshtein, 1993; Millhauser et al., 1995).

Spin labels have been used to characterize the effect of anesthetics on lipid bilayer fluidity (Firestone et al., 1994) and on the properties of spin-labeled membrane proteins. For example, the extracellular domain of the anion-exchange protein in human erythrocytes has been spin-labeled [with bis(sulfo-N-succinimidyl)doxyl-2-spiro-5'-azela], and the rotational mobility of the probe was reversibly increased by high concentrations of diethyl ether (Cobb et al., 1990). Although this could represent direct anesthetic binding to protein, it was interpreted as being due to changes in surrounding lipid bilayer order. Similarly, enhancement of protein side chain mobility by ethanol, as detected by spectral changes in a covalently attached maleimide spin label to selected cysteine residues on the nAChR, is consistent with a change in lipid dynamics (Abadji et al., 1994). Also in the spin-labeled nAChR, isoflurane and hexanol were unable to displace the neighboring lipid molecules in the bilayer from their association with the receptor (Abadji et al., 1993), an observation inconsistent with the lipid protein interface being a binding site for these molecules. Finally, spin labels have been attached to both lipid and protein components of the skeletal muscle sarcoplasmic reticulum Ca²⁺-ATPase in an attempt to examine the mechanism of diethylether activation of

ATPase activity. Again, an increase in the mobility of both the ATPase and surrounding lipid spin labels in the presence of 0.5 M diethylether (Bigelow and Thomas, 1987) was observed and correlated with an increase in activity. It was hypothesized that the increase in spin-label mobility reflected an overall increase in protein dynamics and/or flexibility, which facilitated enzyme activity. Further studies with carefully positioned electron spin resonance probes, combined with other types of spectroscopy, or some form of structural analysis, should allow insight into the influence of anesthetics on target dynamics but may not directly reveal binding locations for reasons already mentioned. Being in some respect similar to site-directed mutagenesis, inclusion of these bulky extrinsic probes into positions previously occupied by groups of different character could have substantial effects on protein structure or dynamics (Calciano et al., 1993), making any subsequent interpretation of anesthetic influences difficult.

e. INFRARED SPECTROSCOPY. Infrared radiation includes wavelengths between 0.7 and 500 μm , or in wavenumbers (number of waves in 1 cm), between 14,000 and 20 cm^{-1} . This part of the electromagnetic spectrum consists of low-energy photons that are ideal for probing the weak interactions thought to be of importance to anesthetic-macromolecule interactions. Infrared radiation is absorbed by the bending and stretching vibrations of various functional groups, resulting in a complex absorption spectrum. This form of spectroscopy has the advantage of rapid time resolution (10^{-12} seconds), allowing discrimination of environments and events not possible with conventional NMR approaches.

Caughey and coworkers have used infrared spectroscopy to characterize the binding environment of nitrous oxide in a variety of protein targets. They examined the shift in the wavenumber position of a specific antisymmetric stretch frequency (ν_3) of N_2O close to 2230 cm^{-1} , which reflects the polarity of the environment of the anesthetic molecule (Gorga et al., 1985; Dong et al., 1994). Two distinct environments for N_2O were found in human and BSA (Hazzard et al., 1985; Dong et al., 1994), one near a benzene-like structure and the other in a more alkane-like environment, consistent with the general features of anesthetic binding sites identified with the other approaches mentioned in this section. Although current work has been limited to N_2O because of specific advantages for infrared signal-to-noise problems, this approach should also be useful for examining the interactions of other anesthetics with proteins. Importantly, because the reporter groups are intrinsic to the system, and are located on all components of the system (anesthetic, target, and solvent), many interactions in the native, unperturbed state can be explored with infrared spectroscopy. For example, the sulfur-hydrogen (S-H) vibration band of cysteine thiols is sensitive to its environment. The bandwidth is indicative of local backbone mobility, and the frequency reflects the

strength of the S-H bond (Alben et al., 1974; Dong and Caughey, 1994). Thus, whereas binding of N_2O to human serum albumin had no effect on the intensity of the amide I band (no change in protein secondary structure), an increase in the intensity of the S-H band of Cys34 at 2563 cm^{-1} suggested subtle tertiary structural alterations in the protein (Dong et al., 1994).

The ability to interpret the infrared spectrum of proteins at the individual bond level is generally impractical because of the large number of overlapping bonds (Mantele, 1993). However, the approach may be applicable to the small synthetic bundle proteins (Gibney et al., 1997a,b) that are being used to define the structural features of volatile anesthetic binding sites (Johansson et al., 1996, 1997) because of their low molecular weight and limited amino acid composition. Furthermore, the examination of the carbon-halogen infrared absorption bands may provide information on the anesthetic atoms that are interacting with the protein.

3. *Direct photoaffinity labeling.* A recent approach developed in our laboratory is halothane direct photoaffinity labeling (Eckenhoff and Shuman, 1993; El-Maghrabi et al., 1992). As previously discussed (see Section II.A.), the rapid kinetics of halothane equilibrium binding preclude or complicate the use of several conventional methods for determining binding location. These rapid kinetics, however, can be converted to a stable covalent bond by cleaving the carbon-bromine bond of halothane with ultraviolet light to produce a reactive chlorotrifluoroethyl radical, which then covalently attaches to residues in its equilibrium-bound environment to form an adduct, probably through a two-step reaction process, should a suitable group be immediately available. If not, as in the case of halothane photolysis in water, the radical prefers to recombine with the free bromine atom, reforming the parent halothane molecule. An ability to recombine with the bromine atom makes it likely that sites of higher affinity will be preferentially labeled over those of lower affinity. After covalent linkage, the adduct location can be identified by including a radioactive atom, such as ^{14}C on the trifluoromethyl carbon, or ^3H on the 1-carbon, or by monitoring regional protein mass with mass spectrometry (Lindeman and Lovins, 1976; Grenot et al., 1994). Importantly, photoaffinity labeling is one of the few methods available for these small anesthetic ligands capable of separating specific from non-specific binding, because unlabeled halothane can be used as a competitor. Accordingly, most halothane labeling of biological membranes was found to be specific, whereas labeling of the lipid portion alone (liposomes) was substantially less inhibited by unlabeled halothane (Eckenhoff, 1996b). Photolabeling has also permitted the mapping of halothane binding sites in soluble proteins, such as serum albumin. Two specific binding sites were identified in BSA (Eckenhoff, 1996a), and these correspond roughly to the location of the two tryptophans in this protein, in agreement with the tryptophan

quenching studies (Johansson et al., 1995a). In accordance with the rapid kinetics and relatively small size of the parent molecule, halothane, the label is found bound to several amino acid residues in the large IIA cavity (Trp214–Arg219). Further, despite clear competition between fatty acids and halothane (Dubois and Evers, 1992; Eckenhoff and Shuman, 1993) for binding to BSA, the photolabeled residues do not coincide with the presumed fatty acid binding domains (Carter and Ho, 1994), indicating allosteric communication between these sites. This is consistent with the global conformational changes that albumin undergoes on binding fatty acids (Carter and Ho, 1994) and the large increase in thermal stability (Shrake and Ross, 1988). Also, the dissimilarity of fatty acid and halothane binding sites is consistent with the low-affinity ($K_d > 5$ mM) interactions of other fatty acid binding proteins and halothane (Evers et al., 1995).

Photolabeling has allowed the initial assignment of halothane binding domains in membrane proteins, as well. In the ligand-gated ion channel, nAChR, from *Torpedo nobiliana* (Eckenhoff, 1996b), halothane photolabeling revealed that each subunit of the pentameric complex is saturably labeled to a similar degree. On digesting the α -subunit with V8 protease (endoprotease Glu-C), >90% of the label was found in the fragments known to contain the four putative transmembrane sequences (approximately half the mass). The distribution of label within these domains is not yet clear but could have significant implications for the mechanism of anesthetic action on these ligand-gated ion channels. For example, preferential labeling of the probable channel-lining helix (M_2) would implicate a channel blocking mechanism, as a recent site-directed mutagenesis study suggests (Forman et al., 1995; see Section II.D.5.). Interestingly, the inability of another inhalational anesthetic, isoflurane, to inhibit halothane photolabeling at reasonable concentrations suggests that protein binding sites for different anesthetics are distinct. Thus, photolabeling results in this member of the ligand-gated ion channel family suggest that there are many specific halothane binding sites, and the majority may be found in the transmembrane domain in a distribution that differs somewhat from the lipid distribution. Such data hint at the importance of these functionally crucial transmembrane regions (lipid-protein or interhelical interfaces) for anesthetic binding and possibly action. Other evidence also supports the lipid-protein interface of membrane proteins as a favored binding site for anesthetic-like molecules (Fraser et al., 1990; Jørgensen et al., 1993; Nakagawa et al., 1994).

Although photolabeling has emerged as a potentially useful tool for the direct determination of both the location and character of halothane binding sites, the photochemistry involved is complex and incompletely characterized. That labeling is the result of ligand activation (photoaffinity labeling) as opposed to protein activation

(site-directed photochemical coupling) (Bouchet and Goeldner, 1997) is best demonstrated by conformational dependence of labeling in peptides with no absorbance at the wavelength used for photolysis (Johansson and Eckenhoff, 1996). Another concern is that the label distribution could be at least partially due to the diffusional range, or chemical selectivity of the chlorotrifluoroethyl radical, as opposed to the parent molecule, halothane. However, the short half-life of the halothane photolysis product relative to halothane off-rates (Eckenhoff and Shuman, 1993) make this unlikely. Finally, the identification of discrete sites in albumin that match those predicted by fluorescence quenching studies, and the conformational dependence of labeling in both serum albumin and PLL, are not consistent with these photochemical concerns as being dominant problems.

4. *Crystallographic approaches.* a. X-RAY DIFFRACTION. This method requires production of suitable three-dimensional crystals of identically oriented protein molecules to allow amplification of the X-rays diffracted by the protein electrons. Such crystals are difficult to produce and have until recently been limited to the water soluble proteins. It is important to realize that the crystal structure may not reflect the native conformation of a protein in the biologically relevant dilute aqueous state (Urbanova et al., 1991; Finn et al., 1995; Kleerekoper et al., 1995; Svergun et al., 1997). Further, the assumptions and algorithms for reducing the map of electron density to a three-dimensional structure involve a degree of subjectivity (Kleywegt and Jones, 1995; Kleywegt et al., 1996). Finally, the low-energy interactions typified by anesthetic binding events may be significantly altered by the forces implicit in the crystallization process. Nevertheless, X-ray diffraction analysis of proteins is currently considered the gold standard for comparison, and has resulted in substantial insight into protein structure/function relationships and protein-ligand interactions (Eisenberg and Hill, 1989; Lewis et al., 1996).

There are few examples of this approach being used to study anesthetic-protein interactions. The binding of xenon (minimal anesthetic concentration ~ 0.7 atm) to crystals of myoglobin has provided the highest resolution data. Using 2.5 atm of xenon and X-ray diffraction analysis of sperm whale myoglobin, this weak anesthetic was noted to bind via van der Waals interactions, at a single site equidistant from the proximal histidine and one of the pyrrole rings of the heme moiety (Schoenborn et al., 1965). Cyclopropane and dichloromethane were also found to bind to metmyoglobin in this site (Schoenborn, 1967; Nunes and Schoenborn, 1973). Interestingly, there is no obvious access pathway from the solvent to this proximal heme site in the interior of the myoglobin molecule for solutes as large as Xe or cyclopropane. Molecular dynamics simulations have resolved this dilemma by revealing transient deviations from the average crystal structure, opening pathways for ligand entry

and exit (Tilton et al., 1988; Daggett and Levitt, 1993). These conformational deviations caused only a 3 to 4% change in overall protein volume but created small cavities with lifetimes of 1 to 20 picoseconds. More binding sites (nonspecific?) in myoglobin could be recruited by raising the xenon pressure to 7 atm (Tilton et al., 1984). Although satisfying some of the general criteria for anesthetic binding sites mentioned in this section (internal cavity, predominance of hydrophobic and aromatic residues, with some polar groups), the relative affinities of Xe and cyclopropane for myoglobin are not consistent with their relative anesthetic potencies, suggesting that this site does not adequately represent the character of the "anesthetic" binding site (Miller et al., 1972). Nevertheless, this work is important in that it has clearly confirmed the concept of the binding of weak ligands to the hydrophobic interior of soluble proteins in the vicinity of aromatic structures and the recruitment of binding sites, as well as the dynamic nature of these sites and access routes.

X-ray diffraction analysis has also demonstrated a single binding site for Xe and cyclopropane on each of the α and β chains of hemoglobin (Schoenborn, 1965). Interestingly, these sites are different from the myoglobin sites, being located more peripherally, and Xe was found to make van der Waals contact with valine, leucine, and phenylalanine. These results suggest that there is a degree of promiscuity to the binding of such a simple ligand, capable of interacting only through van der Waals forces. A greater degree of selectivity is expected as the ligand gains more groups that can interact electrostatically, such as halogens and hydrogens. Accordingly, dichloromethane was shown to bind to hemoglobin at three or four distinct sites, each of which lies in an interior hydrophobic site close to aromatic residues such as Trp14 α or Phe71 β , or at the interface between the hemoglobin subunits near Tyr145 (Schoenborn, 1976).

The binding of a slightly larger anesthetic, dichloroethane, to both bacterial haloalkane dehalogenase (Verschueren et al., 1993) and insulin (Gursky et al., 1994) has also been studied by X-ray diffraction analysis. In the former, the dihaloalkane is a native substrate from which the microorganism derives energy. As shown in figure 4, the dihaloalkane binds in an interior hydrophobic site with weak electrostatic contacts between the two chlorines and two tryptophan and two phenylalanine residues. This site will also bind and dehalogenate smaller haloalkanes such as methyl chloride and ethyl chloride, but with lower apparent K_m values, suggesting the importance of the two additional electrostatic contacts with the 2-carbon halogen or loss of optimal van der Waals contacts. It is interesting to note that, even with the tight fit of dichloroethane into this interior cavity (fig. 4), and the multiple halogen-aromatic electrostatic contacts, the K_m for dechlorination of dichloroethane reflects an affinity (0.8 mM) in the same range as found so far for the inhaled anesthetics by direct binding

studies (Dubois and Evers, 1992; Dubois et al., 1993; Eckenhoff and Shuman, 1993; Johansson et al., 1995a).

The insulin dichloroethane binding cavity, on the other hand, is lined by serine, valine, glutamate, and tyrosine and is so small and sterically hindered that only the cis conformation of dichloroethane will bind. All of the clinically used haloalkane anesthetics are too large to bind in this insulin cavity, whereas those that are much smaller, like dichloromethane, also bind poorly, again probably because of the loss of van der Waals contacts. Interestingly, the insulin cavity contains structured water molecules, some of which are displaced on haloalkane binding, suggesting an additional entropic contribution to the binding interaction (see thermodynamic section). Unfortunately, visualization of the small water molecules with X-ray diffraction requires exceptional crystals and very high resolution structures, so sufficient resolution has not been achieved in other protein-anesthetic systems to determine whether this is a general feature of anesthetic binding.

The only X-ray diffraction data that incorporate a clinically important haloalkane anesthetic are that of halothane binding to adenylate kinase (myokinase) (Sachsenheimer et al., 1977). Halothane inhibited the activity of this enzyme with a K_i of 2.5 mM, and only at low adenosine monophosphate concentration (100 μ M), suggesting a competitive interaction with the nucleotide (see Section II.D.1.). X-ray diffraction of crystals soaked in "saturated" solutions of halothane showed localization of the halothane molecule in a discrete interhelical niche, lined by the hydrophobic residues valine, leucine, and isoleucine, but also by the more polar residues tyrosine, arginine, and glutamine (Pai, et al., 1977). Yet again, this site fulfills the criteria suggested above (see Section II.D.) that an anesthetic binding site, although necessarily hydrophobic, should also have some polar (aromatic?) character. This site was also identified as that binding adenosine monophosphate, providing confirmation of the suspected competitive interaction. Interestingly, halothane binding produced no detectable conformational change in adenylate kinase at this low (0.6 nm) resolution. Also, the very high halothane concentration used to soak these crystals may render this site somewhat nonspecific and possibly not even related to the observed functional effect. More such studies at higher resolution with controlled anesthetic concentrations will be required to refine our concepts of the structural features underlying anesthetic-protein interactions.

The study of membrane protein structure with X-ray diffraction analysis has been difficult because of the requirement for two different solvent environments (lipid and water). Some progress, however, has been made with novel crystallographic approaches using short-chain detergents or monoclonal antibody fragments (Deisenhofer and Michel, 1991; Iwata et al., 1995), and with highly ordered natural membrane proteins, such as bacteriorhodopsin (Nakagawa et al., 1994). In the latter

case, low-resolution difference analysis of the electron density maps with and without diiodomethane found the anesthetic to be located in the lipid-filled center of the naturally occurring protein trimers, suggesting lipid-protein interfacial binding. In this system, however, the implication of such a site must be viewed with some caution, because the low lipid-to-protein ratio indicates that most of the lipid molecules are, in fact, interfacial.

b. ELECTRON DIFFRACTION. A related technique that may provide useful structural information on anesthetic interactions with membrane proteins at moderate resolution is electron cryomicroscopy (Chiu, 1993; Dorset, 1995). The structure of proteins that form crystal-like sheets, such as several membrane proteins under native conditions, can produce density maps that allow up to 0.35 nm resolution. For example, this method has been used to define the structure of bacteriorhodopsin and of the nAChR to a resolution sufficient to allow a view of the overall topology of the protein but not to the amino acid level (Henderson et al., 1990). Unwin (1995) combined this approach with clever cryopreparative techniques to show that binding of acetylcholine to the *Torpedo marmorata* nAChR results in subtle allosteric conformational changes in the α -helices that line the pore of this ligand-gated ion channel. Although a general idea of the anesthetic binding location in membrane protein models may be possible, the paucity of suitable crystals and low-resolution, inherent to electron diffraction may limit its usefulness in defining the presumably more subtle influences of the inhalational anesthetics on protein structure.

5. *Molecular genetics.* Another approach to anesthetic-protein interactions that may allow localization of binding sites is provided by molecular genetics techniques. This broad approach can be divided into three levels: (a) identifying the gene product of mutant organisms with altered anesthetic sensitivity, (b) identifying important domains for an anesthetic response by mixing regions of proteins with varied anesthetic sensitivity (chimeras), and (c) identifying amino acids in sensitive proteins that participate in the anesthetic response by site-directed mutagenesis. Examples of all three are currently underway in several laboratories.

Although many interesting examples of the first approach are available, it will be mentioned here only to point out that it has the potential to identify proteins that participate in the behavioral response to anesthetics and does not itself provide any information on anesthetic-protein interactions (McCrae et al., 1993; Sedensky et al., 1994; Morgan and Sedensky, 1994; Campbell and Nash, 1994). However, when interesting gene products are identified, the study of their interactions with anesthetics using the other methods presented here is a reasonable next step and may prove fruitful.

The broadest example of the second approach is the combination of subunits of oligomeric proteins in the same family that have different anesthetic sensitivities in an attempt to determine the subunit specificity re-

sponsible for the anesthetic effect. In the GABA_A receptor/channel complex, this has provided interesting but confusing results that have recently been summarized by Harris and colleagues (1995). Of note was the fact that no single GABA_A subunit (of the many) was consistently necessary for anesthetic potentiation of function, suggesting, as noted above (see Section II.D.3.), that multiple, general binding sites for these drugs exist in these oligomeric membrane proteins. One GABA receptor subtype, however, the homomeric ρ receptor, has a very different anesthetic sensitivity and is currently being used for construction of chimeric receptors in an attempt to map regions of the subunit necessary for anesthetic sensitivity. However, because ρ has low homology (30 to 38%) with anesthetic-sensitive GABA_A channels, replacement of entire regions of the primary structure could have important consequences on the expression, assembly, and global structure of the oligomeric complex. This second molecular genetics approach is often used to hone the focus of the third approach.

The resolution can be increased to the individual residue level by swapping single amino acids in specific areas of a protein using site-directed mutagenesis. In a recent example, specific substitutions in the middle of the putative pore-forming M₂ transmembrane segments of the nAChR (the local anesthetic site) were shown to increase the sensitivity of expressed channels to both inhaled anesthetics and alcohols (Forman et al., 1995). Further, the change in sensitivity roughly correlated with the hydrophobicity of the substitution (serine, valine, isoleucine, phenylalanine). The suggestion that this M₂ site represents a site of anesthetic interaction must be tempered by the knowledge that such amino acid substitutions could alter the oligomeric conformation sufficiently that remote anesthetic interactions may be transduced differently. For example, there is clear evidence for local anesthetics binding to the M₂ region in at least an overlapping distribution with the proposed general anesthetic site (Leonard et al., 1991), and yet the expected competition between local and general anesthetics has not been demonstrable (Dilger and Vidal, 1994). Nevertheless, these data emphasize the importance of these transmembrane regions for anesthetic sensitivity. One means of reducing the ambiguity of such approaches in these very complex oligomeric proteins first may be to study their isolated components. For instance, pentameric helical bundle models of the pore region of the ligand gated ion channels can now be constructed and studied with a variety of the tools already mentioned. Such models are currently being studied to determine fundamental mechanisms of ion channel function (Lear et al., 1988; Kienker et al., 1994). An initial attempt to examine the interactions of synthetic helical bundles with anesthetics found only weak binding ($K_d \cong 3$ mM) of halothane within the hydrophobic core of a water soluble four- α -helix bundle (Johansson et al., 1996), but redesign of the hydrophobic core with the

goal of creating a cavity resulted in improvement of the halothane binding affinity (Johansson et al., 1997).

Molecular genetics is therefore a powerful tool that can identify proteins, domains, and amino acids important for a functional response to anesthetics. Ambiguity with respect to actual sites of interaction will remain, however, unless these approaches are combined with other methods designed to evaluate structure or sites more directly. An excellent example is recent work on the phage T4 lysozyme. Site-directed mutagenesis in various regions of the protein were accompanied by crystallographic analyses, CD spectroscopy, isothermal titration calorimetry, and functional analyses to fully investigate the implications of the mutations in terms of protein structure, stability, activity, and the energetics of ligand binding (Eriksson et al., 1992a,b; Morton et al., 1995; Morton and Matthews, 1995). It was found, for example, that substitution of an alanine for a leucine in the hydrophobic core (Leu99Ala) created a cavity that bound benzene with an apparent K_d of 0.40 mM, remarkably similar to values obtained for halothane binding to albumin. Similar to the albumin-halothane interaction, benzene stabilized the mutant protein (see Section III.B.). Despite this, benzene binding produced very small alterations in the crystal structure, such as main-chain shifts of only ~ 0.05 nm. Although such studies will be difficult to reproduce in membrane proteins, this work is important because it shows that single mutations can produce dramatic changes in stability, function, and ligand-binding character and emphasizes the necessity for caution in the interpretation of experiments employing only functional measures.

6. *Binding site character summary.* There is a very limited database from which to generalize the structural features of an anesthetic binding site in a protein target. However, the available information suggests that cavity volume, shape, and hydrophobic character are all important in creating a binding site for inhalational anesthetics. These features may in part be contributed by the bulky hydrophobic side chains of leucine, isoleucine, tryptophan, and phenylalanine. In addition, polar character is necessary, which appears to assist in the stabilization of the binding interaction and which also may be contributed by the aromatic side chains (dipole and quadrupole moments), or by residues like arginine and glutamine. At this point, discrete binding sites are less apparent in membrane proteins than soluble proteins, and available evidence suggests there are multiple binding sites at the lipid-protein and/or protein-protein interfaces.

III. Structural and Dynamic Consequences of Anesthetic Binding

Anesthetic binding to a protein is a necessary prelude to altering its activity, but the underlying mechanisms of protein dysfunction ultimately involve the conformational or dynamic consequences of binding. Unfortunately, such structural and dynamic changes have not

been widely studied and are difficult to demonstrate. This is not surprising given the early state of our ability to define protein structure and the likelihood that the structural or dynamic effects of anesthetic complexation are small. The tools for defining protein structure at high resolution are only just now being used to define protein-ligand interactions, and the most easily characterized proteins are the small soluble proteins that anesthetics apparently minimally influence. Further, the creative approaches for crystallizing membrane proteins to allow X-ray diffraction analysis, as mentioned above (see Section II.D.4.), may limit the ability to study interactions with ligands, especially ligands with weak interactive capability.

Nevertheless, the independent observations that inhalational anesthetics bind to protein and then alter protein activity indicate that a change in protein conformation or dynamics must be occurring. Even an interaction characterized as competitive is likely to be accompanied by such changes, because it is clear that small changes in protein conformation, and more substantial changes in stability and dynamics, occur with the binding of native ligands (e.g., fatty acids and albumin). We will briefly discuss the consequences of anesthetic binding to each level of protein structure, recognizing that substantial overlap between these areas must exist. The brevity of this section is the unavoidable consequence of the limited information available.

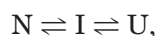
A. Secondary Structure

Hydrogen bonding between the carbonyl oxygen and the amino nitrogen of the peptide backbone (main chain) is responsible for the various elements of secondary structure: α -helix, β -sheet, loops, and turns. Secondary structure is a vital element of the ultimate protein structure in that it may control or serve as a nidus for folding. Therefore, any disruption of secondary structure through interference with hydrogen bonds, for example, will have profound effects on protein structure and function. As mentioned previously (see Section II.C.2.), this may be relevant to the inhalational anesthetics because of their predicted ability to hydrogen bond, either as an acceptor or donor, in the hydrophobic interior of proteins. An extension of the proposition that anesthetic binding forces are contributed by atypical hydrogen bonds is that the hydrogen-bonding ability may be sufficiently strong to disrupt or reduce the stability of native peptide hydrogen bonds. Such "competitive" hydrogen bonding could have widespread effects on protein conformation through destabilization of secondary structure. However, such widespread influences have been difficult to demonstrate. Ueda and coworkers (Shibata et al., 1991; Chiou et al., 1992) reported a slight shift from α -helix to β -sheet for PLL in the presence of anesthetics, using Fourier transform infrared and CD spectroscopy (Shibata et al., 1991; Chiou et al., 1992), but in more recent experiments with the same ho-

mopolymer, we were unable to observe any significant change in secondary structure with up to 12 mM halothane (Johansson and Eckenhoff, 1996). Also, no change was observed in the secondary structure of the highly α -helical protein, albumin (Johansson et al., 1995a), or in a four- α -helix bundle protein (Johansson et al., 1996) as determined by CD spectroscopy in the presence of 12 mM halothane (almost two orders of magnitude greater than clinical EC_{50}), even though significant binding occurs. Although these results suggest that anesthetics cannot interfere with main chain hydrogen bonds, they do not necessarily rule out a hydrogen bonding interaction between protein and anesthetics. For instance, crystallographic data indicate that between 5 and 24% of the potential hydrogen-bonding groups in proteins are unpaired (Savage et al., 1993; McDonald and Thornton, 1994). Such unfulfilled hydrogen-bonding groups may find suitable partners in the anesthetic molecule, possibly explaining binding and the increased stability observed in albumin (see Section II.B.).

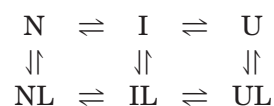
B. Tertiary Structure

The tertiary structure of proteins is the result of poorly understood folding processes of secondary structural elements, which in some cases are aided by chaperone proteins (Gething and Sambrook, 1992). Although controversial, the predominant mechanism for folding is probably the seclusion of hydrophobic regions from water, although it is also clear that electrostatic (hydrogen-bonding) forces are involved (Lazaridis et al., 1995; Makhatadze and Privalov, 1995). It is important to realize that the ultimate folded conformation of most proteins is only marginally more stable (<5–10 kcal/mol) than the unfolded form, resulting in a dynamic equilibrium between the folded and unfolded state (Pace et al., 1996). For most biological proteins, this equilibrium is likely to include one or more intermediate folded states. Therefore, to understand and model this further, it is necessary to increase the complexity of the anesthetic target as follows:



where N is the native, fully folded state of the protein, I is one of perhaps many folding intermediates, and U is the fully unfolded form. Depending on the stability of the native state, the rate constants are such that the I and U forms of the protein are poorly populated under normal conditions. This situation can, however, be altered by many factors, including temperature, pH, salt concentration, different solvents, and the presence or absence of ligands. With apomyoglobin, for example, the maximally populated state is the fully folded native form at pH 6.5, a partially unfolded form at pH 4.5 and the fully unfolded form at pH 2.5 (Loh et al., 1995). The addition of heme stabilizes the native, folded form of this protein (now holomyoglobin), so that the pH effect on stability is

substantially reduced. The effect of ligand binding on the conformational equilibrium can be modeled as:



For myoglobin, stabilization by the native ligand heme implies a preferential binding to the native, folded form of apomyoglobin, resulting in a shift of the folded/unfolded equilibria to predominately populate the liganded, or holomyoglobin form (in this case, NL). This is reflected in a larger ΔG° and therefore a greater resistance to unfolding by chemical or thermal means. Stabilization of the native folded state may be true of high-affinity ligands in general, whereas compounds that bind with considerably lower affinity, such as the chaotropic salts (urea or guanidine), tend to shift the equilibrium toward the unfolded form (UL) because of multiple favorable binding interactions with the exposed amides or other nonspecific sites in the U conformation (Monera et al., 1994). It is interesting to note that even high-affinity ligand-protein interactions, which result in large shifts in the folded/unfolded equilibria and dramatic stabilization of the folded conformation, tend to produce only small changes in the average three-dimensional structure of the protein as represented by X-ray diffraction of solid protein crystals (Kurumbail et al., 1996; Laskowski et al., 1996). As stated by Jardetzky (1996), "... the transmission of conformational information may not depend on changes in average structure, but on a modulation of protein dynamics."

This implies that proper assessment of the consequences of anesthetic binding to protein requires methods to examine both the average spatial relationships between important elements (X-ray crystallography, NMR) as well as the dynamics and stability. Little data are available for the inhaled anesthetics in either case. As mentioned above (see Section II.D.1.), changes in native ligand binding in the presence of anesthetics (e.g., benzodiazepine binding by serum albumin) are most likely due to competition of the two ligands for different conformational states of the target. Functionally, this would be apparent as competition, typically interpreted as interaction within the same site. For example, anesthetic competition with the native substrate for firefly luciferase in functional studies has been repeatedly interpreted as indicating interactions within the same site (Franks and Lieb, 1984). Thermal stability experiments, on the other hand, strongly suggest that the observed competitive kinetics are the likely result of competition for binding to different conformational states of luciferase (Chiou and Ueda, 1994). Luciferin and known competitive antagonists stabilize this protein against thermal unfolding, implying preferential binding to the native, folded form, whereas anesthetics (and alcohols) destabilize the enzyme (Chiou and Ueda, 1994), suggest-

ing preferential binding to either the unfolded form or a partially folded intermediate. Again, the favored conformational state of luciferase or other proteins may be only subtly different from the native state, especially with regard to the average structure, in accordance with the small local changes found in the X-ray crystal structure of the few proteins examined with bound anesthetic (xenon, cyclopropane, dichloromethane, dichloroethane, benzene). Further, structural differences may not be confined to the immediate neighborhood of the anesthetic binding site. The binding of fatty acids by serum albumin causes backbone shifts throughout the protein's structure (Carter and Ho, 1994), and even small ligands like 1,2-dichloroethane caused a small displacement of sulfate ions from distant sites on binding to insulin crystals, apparently through allosteric conformational changes (Gursky et al., 1994). Thus, such uncertainties and apparent discrepancies serve to emphasize the need for complimentary approaches to the structural consequences for the protein of anesthetic binding.

Although the structural effects of anesthetic binding are probably small and are therefore difficult to demonstrate, anesthetics could have a more marked effect on protein dynamics. This implies that approaches capable of discerning protein motions and stability will be necessary to characterize the important effects of the inhaled anesthetics. Although X-ray diffraction of protein crystals is typically characterized as providing only static information, the method also may yield dynamic information via the average atomic thermal coefficients, or Debye-Waller factors. This factor represents uncertainty of atomic position, and its dependence on temperature has been considered a reflection of motion (Tilton et al., 1992). Similarly, heterogeneous uncertainty within three-dimensional protein structures obtained with NMR spectroscopy provides vivid evidence for the dynamic nature of macromolecules in solution (Jardetzky, 1996). In addition, NMR spectroscopy can be used to probe the motions of specific amino acid side chains in proteins (Ernst, 1992; Ernst and Ernst, 1994). In discussing the anesthetic location, we have already presented the use of some approaches that monitor dynamics of the immediate environment of specific reporter groups, such as fluorescence anisotropy and electron paramagnetic resonance. In addition to the previously mentioned limitations that such spectra re-

flect either local or global effects of the interaction, it bears emphasis that each of these approaches samples a different portion of the dynamic time scale, and the consequences of a given protein-ligand interaction may only be apparent within a narrow region of the time domain. Table 2 gives the approximate time scale during which specific molecular events occur and the appropriate approach to access these events.

An additional technique for examining protein dynamics is the measurement of hydrogen exchange kinetics (Englander and Englander, 1994). As protein structure fluctuates, amide hydrogens become transiently exposed to solvent (water) hydrogens and undergo exchange reactions with a rate that depends on pH, temperature, the character of the residue side chain, and the secondary structure. The overall rate of exchange becomes a function of the rate that the protein populates progressively more unfolded states and can therefore be used as a measure of protein dynamics. The events that expose amide hydrogens can be local fluctuations in structure, or more global (but less frequent) unfoldings, but would in general be classified as falling into the rigid body motion or global unfolding time domain (10^{-3} seconds to minutes or hours). Exchange kinetics can be followed by measuring either uptake or elimination of tritium, using scintillation counting, or deuterium, using mass spectrometry or NMR. Some spatial resolution can be achieved by combining hydrogen-tritium exchange with rapid proteolysis, or combining hydrogen-deuterium exchange with two-dimensional NMR spectroscopy. An advantage of hydrogen-exchange measurements is that the dynamic time domain sampled can be manipulated by a factor of over 10^6 , independently of protein dynamics, by varying the exchange chemistry through alterations of pH and temperature.

One particularly promising avenue for the exploration of more rapid events, molecular dynamics simulations, has taken advantage of the rapid advances in computer processing capability and statistical mechanics to produce accurate simulations of very complex systems for up to a few nanoseconds (Karplus and Petsko, 1990; Tuckerman et al., 1996). As stated previously, simulations of halothane in water have defined the hydration shell of halothane and have questioned the ability of the sole halothane hydrogen to participate in hydrogen bonds (Scharf et al., 1996). The only reported example of

TABLE 2
Protein motions, dynamic time domains, and sampling methods

Motion	Example	Time domain (sec)	Method
Vibrational	Atomic fluctuations	10^{-15} to 10^{-6}	Infrared, fluorescence, molecular dynamics simulations
Side chain	Ring flipping	10^{-12} to 10^1	Fluorescence anisotropy, EPR, NMR, near UV circular dichroism
Rigid body	Hinge movement	10^{-6} to 10^2	NMR, X-ray crystallography, hydrogen exchange
Global	Protein unfolding	10^{-3} to 10^3	DSC, hydrogen exchange, far-UV circular dichroism

Adapted from Brooks et al. (1988) and Jardetzky (1996).

DSC, differential scanning microcalorimetry; EPR, electron paramagnetic resonance; NMR, nuclear magnetic resonance; UV, ultraviolet.

simulations of an anesthetic interaction with a more complex system is that of trichloroethylene and polar lipids (Huang et al., 1995), which concluded that the anesthetic localized in the acyl chain region close to the polar head group. In another theoretical study, Pohorille and coworkers (1996) found that an energy minima exists for anesthetic localization just inside the water-lipid interface, whereas for similar but nonanesthetic molecules, such a minima did not exist. It may be possible in the near future to characterize all components of anesthetic protein interactions at the atomic level on the 10^{-12} to 10^{-8} seconds time scale, something not currently possible with any form of spectroscopy or crystallography.

Protein dynamics can be indirectly discerned in a somewhat less visual way: through measurements of protein stability. The relationship between protein stability and function is complex and poorly understood, but function is predicted to require some degree of conformational flexibility (Frauenfelder et al., 1991; Rasmussen et al., 1992). This is illustrated by the effect of temperature, whereby limited elevations often enhance protein activity but also decrease stability by increasing the conformational entropy of the protein. In addition, modifications in protein structure that increase stability can be associated with a decrease in function (Lim et al., 1994). On the other hand, the liganded form of many proteins, also a requirement for activity, is often associated with an increase in stability (Sturtevant, 1987). Thus, considerable insight into the mechanism of anesthetic action on protein function may be gained by comparing the effect of anesthetic and native ligand binding

on protein stability. Stability may be measured by determining the concentration of chaotropic salt (guanidine or urea) necessary to unfold the protein as reflected by α -helical content, or by measuring heat capacity as a function of temperature (differential scanning microcalorimetry). Accordingly, the insight into the mode of anesthetic action on firefly luciferase reported by Chiou and Ueda (1994) and discussed earlier in this section, was generated through differential scanning microcalorimetry experiments. Although Ueda generalizes destabilization as a consequence of anesthetic interaction with all proteins, preliminary data from our laboratory (Tanner et al., 1997) suggest that, like the benzene-T4 lysozyme interaction (Morton et al., 1995), serum albumin is rendered substantially more stable by halothane (fig. 5), suggesting that halothane binds preferentially to the native folded form of the protein. This latter result is similar to the effect of fatty acids on albumin (Shrake and Ross, 1988), is consistent with the observed enthalpic contribution to halothane binding in discrete albumin sites (fig. 1), and suggests that anesthetic-induced protein dysfunction may proceed by disparate mechanisms. Perhaps such differential binding to different conformational states of proteins underlies the curious ability of anesthetics to increase the activity of some proteins and decrease that of others.

It is as yet difficult to reconcile changes in dynamics and stability with a general model of anesthetic mechanisms, and such attempts should be viewed with caution. Nevertheless, some interesting observations can be made. For example, 50 to 100 atm pressure is known to antagonize anesthesia (Wann and MacDonald, 1988)

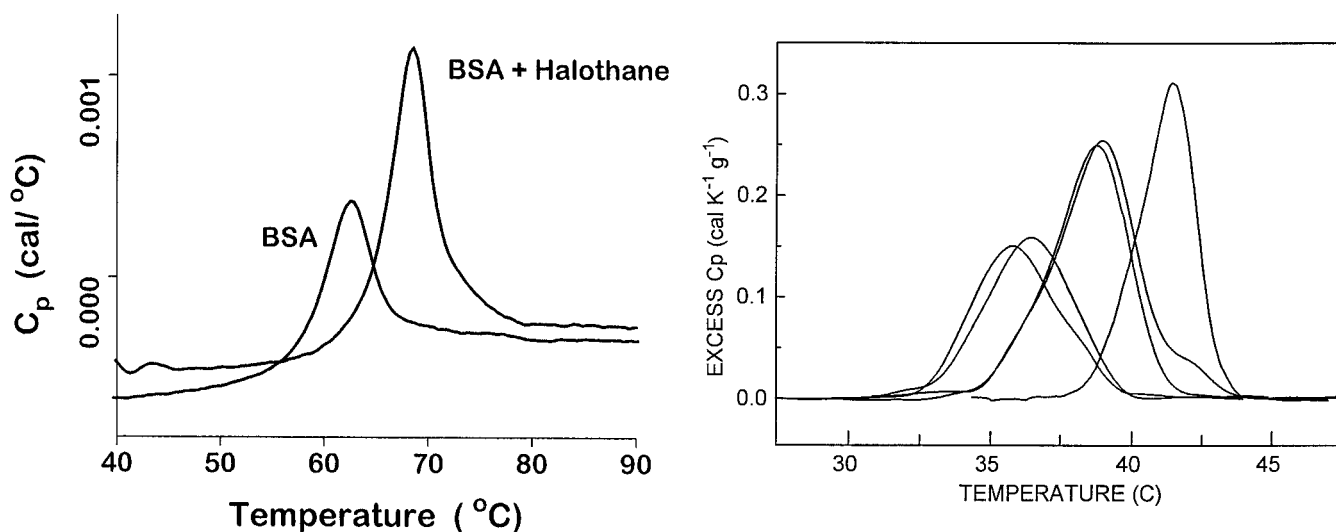


FIG. 5. Differential scanning calorimetry of BSA (a) or firefly luciferase (b) with and without anesthetics. The thermal transition (unfolding) of BSA is shifted to higher temperatures by approximately 5 mM halothane (stabilization), whereas the anesthetics shift the transition of luciferase to lower temperatures (destabilization). (b) The curves are (left to right) halothane, chloroform, enflurane, isoflurane, and control. Each anesthetic was present in approximately 3-mM concentration. For both stabilization and destabilization, the influence on transition temperature was concentration-dependent. Generally speaking, stabilization indicates that the anesthetic is preferentially binding to the native, folded form of the protein, whereas destabilization indicates preferential binding to either the unfolded form or a folding intermediate. Panel b kindly provided by Dr. Issaku Ueda from the University of Utah.

and also to reduce the stability of most proteins (Weber and Drickamer, 1983) because of a lower apparent molecular volume in the unfolded state. At the molecular level, then, pressure would be predicted to antagonize stabilization and enhance destabilization, suggesting that stabilizing events in some proteins contribute to the anesthetic state. Likewise, a decrease in body temperature increases 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 result of anesthetic-protein interactions. Finally, the higher affinity of the binding interactions that result in stabilization (e.g., BSA) is closer to the concentration required to produce anesthesia in animals (~0.2 to 0.4 mM) than those resulting in destabilization, although the necessary magnitudes of stabilization, the domains stabilized, or the proteins stabilized are by no means clear at this point.

C. Quaternary Structure

There are numerous examples of signaling proteins potentially relevant to anesthetic action that are oligomeric. Proper assembly and concerted interaction of the subunits of these complexes governs their function, and these subunits interact principally through hydrophobicity. Further, because protein-protein interfaces are characterized as having the largest cavities (Hubbard and Argos, 1994), they are a particularly attractive candidate for an inhalational anesthetic binding site. The result of anesthetic binding in such a site may have important consequences to the function of the complex. It could stabilize subunit interactions so that separation, important in receptor-G-protein activity, for example, is slowed. This has been suggested as a general explanation for the inhibitory effect of inhalational anesthetics on activity of the muscarinic acetylcholine receptor (Aronstam and Dennison, 1989). On the other hand, anesthetic binding at protein-protein interfaces might weaken the association between subunits that are important for the activity of, for example, the ligand-gated ion channels. Allosteric binding may also weaken association, because subunits and anesthetics may compete for different conformational or dynamic states of the target. Attenuation of subunit association may also promote oligomeric protein function, as in the case of the sarcoplasmic reticulum Ca^{2+} -ATPase. It has been found, for instance, that halothane and diethylether activate function and simultaneously reduce dimerization of the Ca^{2+} -ATPase monomers (Bigelow and Thomas, 1987; Karon and Thomas, 1993), presumably by binding to interfacial sites. Finally, the lipid-protein interface, already identified as a potential site for anesthetic binding, may be viewed as a special sort of quaternary structural element and thus could contribute to the anesthetic action in the same manner.

In summary, structural and dynamic information on anesthetic-protein interactions is very limited, but the weight of evidence indicates that subtle changes to both structure and dynamics are occurring. The recent emergence and continued refinement of experimental and theoretical tools for determining protein structure and dynamics offer a challenging opportunity to define anesthetic action at this most fundamental level.

IV. Summary

The fundamental interactions of inhalational anesthetics with proteins have been considered in some detail, using specific examples where appropriate to illustrate these interactions and demonstrate progress. It is now clear that these low-affinity volatile molecules with rapid kinetics can specifically bind to discrete sites in some proteins at reasonable pharmacological concentrations, and some general features of these sites are beginning to emerge. The structural or dynamic consequences of anesthetic binding, however, are still vague at best. The remaining challenge is to define which interactions produce anesthetic binding to relevant targets and what the features of this relevant anesthetic binding site are. Finally, and most importantly, how does the occupancy of these pockets, patches, or cavities result in the subtle alterations in protein conformation and dynamics that confound their function and ultimately produce the behavioral response that we term "anesthesia"?

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