Predictability of Weak Binding from X-ray Crystallography: Inhaled Anesthetics and Myoglobin†

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ABSTRACT: Xenon and dichloromethane are inhalational anesthetic agents whose binding to myoglobin has been demonstrated by X-ray crystallography. We explore the thermodynamic significance of such binding using differential scanning calorimetry, circular dichroism spectroscopy, and hydrogen−tritium exchange measurements to study the effect of these agents on myoglobin folding stability. Though specific binding of these anesthetics might be expected to stabilize myoglobin against unfolding, dichloromethane actually destabilized myoglobin at all examined concentrations of this anesthetic (15, 40, and 200 mM). On the other hand, xenon (1 atm) stabilized myoglobin. Thus, dichloromethane and xenon have opposite effects on myoglobin stability despite localization in comparably folded X-ray crystallographic structures. These results suggest a need for solution measurements to complement crystallography if the consequences of weak binding to proteins are to be appreciated.

How inhaled anesthetic agents alter protein function is poorly understood. Membrane proteins, such as neurotransmitter receptors, in the central nervous system are currently favored targets of anesthetic action (*1*). However, structural techniques with atomic-level resolution such as X-ray crystallography have not yet been successfully applied to most such proteins. Nevertheless, inhaled anesthetic agents probably interact with many proteins, both membrane and soluble, and we can learn the underlying principles from selected examples (*2*). Indeed, X-ray crystallography has provided direct structural evidence for interactions between inhaled anesthetic agents and several soluble proteins (myoglobin, adenylate kinase, firefly luciferase, and serum albumin) not likely to be responsible for anesthetic action (*3*−*7*).

Myoglobin was the first protein whose structure was determined by X-ray crystallography (*8*), and, in a subsequent study, myoglobin crystals were mounted in the presence of inhaled anesthetic agents, in part to investigate binding sites for these small molecules. A binding site for some anesthetics, such as xenon, cyclopropane, and dichloromethane, was identified in the interior of myoglobin, although other anesthetic molecules, such as ethane, were reported not to bind (*4*). It remains unclear, though, whether the dominant effect of inhaled anesthetic agents on proteins is due to specific interactions, such as those demonstrated by crystallography, or nonspecific interactions, such as hydrophobic interactions without particular protein structural requirements. We have addressed this question with stability measurements.

Specific ligands generally stabilize proteins against unfolding (*9*), as long as there is more Gibbs energy of binding to the native, folded protein state than to the unfolded state (*10*). The Gibbs energy of ligand binding to either state is determined by the number of binding sites and the association constants, as well as the concentration of free ligand (*10*). In the absence of binding to the native state, and ignoring intermediate conformers for the present, nonspecific interactions with the unfolded state will destabilize a protein and enhance unfolding. We previously demonstrated that halothane, a halogenated ethane, enhances thermally induced unfolding (*11*) and decreases protection factors for slowly exchanging hydrogens (*12*) of myoglobin. This may be attributed to the general tendency (*13*) of hydrophobic compounds to unfold proteins and the absence of specific halothane binding sites in the folded state of myoglobin. We also demonstrated that, in contrast, halothane stabilizes serum albumin (*12, 14, 15*), a protein whose native state binds halothane saturably (*16*−*18*). Thus, we predicted that the smaller molecules previously shown by X-ray crystallography to have binding sites in the native folded state of myoglobin would stabilize this protein against unfolding. We tested this prediction by evaluating the effect of dichloromethane and xenon on myoglobin stability, and found opposite effects of the two ligands.

Dichloromethane and xenon have both been used as anesthetic agents, though neither is currently in as widespread clinical use as halothane, isoflurane, desflurane, and sevoflurane. Nevertheless, a good explanation of how any of these anesthetic agents interact with proteins should include the simpler molecules, dichloromethane and xenon. Dichloromethane vapor was introduced as an anesthetic agent in the nineteenth century, and was used in the early twentieth

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century (*19*−*21*). The partial pressure for anesthesia is reported to be 0.03 atm[1] dichloromethane (*22*). Xenon gas was shown to anesthetize human beings in 1951 (*23*), at a partial pressure of 0.71 atm (*24*), and this has recently received renewed interest, both from clinical (*25, 26*) and from mechanistic (*27, 28*) perspectives.

MATERIALS AND METHODS

Myoglobin from horse skeletal muscle was obtained from Sigma Chemical Co. (St. Louis, MO, product number M-0630) in powdered form and dissolved in 20 mM KH_2PO_4, 150 mM NaCl (pH adjusted to 7.0 with HCl) buffer. A solution of recombinant sperm whale myoglobin was also obtained from Sigma (product number M-7527). Dichloromethane (99.9% A.C.S. HPLC grade, CH_2Cl_2, also called methylene chloride) was obtained from Sigma (product number 27,056-3). Xenon (grade 5.0) was obtained from BOC Gases (Riverton, NJ).

For the experiments with 15, 40, or 200 mM dichloromethane, the appropriate volume of this liquid was injected directly into a 5 mL gastight syringe (Hamilton Co., Reno, NV) containing the experimental concentration of myoglobin in buffer with a magnetic stirring bar. The gastight syringe was then capped and placed on a magnetic stirring plate for at least half an hour. The aqueous solubility of dichloromethane is about 150 mM at 25 °C (*29*), so our "200 mM" condition was essentially saturated. For the experiments with xenon, xenon gas was flowed over the myoglobin solution in a test tube with gentle agitation for at least an hour.

Stability was assessed by differential scanning microcalorimetry, circular dichroism spectroscopy, and hydrogen−tritium exchange. High-sensitivity differential scanning calorimetry was performed using an MCS microcalorimeter (MicroCal Incorporated, Northampton, MA). The calorimetry cells were heated from 30 to 95 °C, at a rate of 2 °C/min, and heat capacity data were collected with a filter period of 5 s. During scans, cells were pressurized with 1−2 atm of nitrogen, as recommended by the manufacturer, to prevent formation of gas bubbles. Baseline stability was enhanced by prerunning several scans with plain buffer in the sample and reference cells until thermal equilibration was achieved. The experimental sample was then loaded into the sample cell and scanned. (The protein solution was degassed prior to addition of anesthetic agent.) The last buffer−buffer scan of the preexperimental series was used as the instrument baseline for subtraction from experimental records before analysis. The temperature at peak heat capacity, T_m, provides a measure of protein stability. (For reversible unfolding, T_m is generally taken to be the unfolding midpoint temperature, which differs from the point of maximum heat capacity by a fraction of a degree.) Protein concentrations for calorimetry experiments were 2 mg/mL, verified by absorption spectroscopy using the values of 3×10^4 M^{-1} cm^{-1} for the extinction coefficient at 280 nm and 17.6 kDa for molecular mass.

For the circular dichroism experiments, ellipticity at 222 nm was measured at 2 °C intervals over the temperature range from 60 to 94 °C for the control and 40 mM dichloromethane conditions and from 30 to 90 °C for the 200 mM dichloromethane condition. The bandwidth was 1 nm, the temperature equilibration time was 2 min, the measurement time was 10 s, and the protein concentration was 0.16 mg/mL. The data of ellipticity (Θ) vs temperature (T, in degrees kelvin) were fitted to a curve of the form $\Theta = \{E_n + m_n \cdot T + (E_u + m_u \cdot T) \cdot \exp[\Delta H \cdot (T/T_m - 1)/RT]\}/[1 + \exp[\Delta H \cdot (T/T_m - 1)/RT]]$, where R is the gas constant; this corresponds to a two-state, reversible transition at temperature T_m, with enthalpy change, ΔH, and linear pre- and posttransition baselines as described previously (*15*). The change in free energy for unfolding ($\Delta\Delta G$) can be calculated from the change in T_m (denoted ΔT_m) using the formula $\Delta\Delta G = \Delta H \cdot \Delta T_m / T_m$ (*30*).

For the hydrogen−tritium exchange measurements, protein solutions (10 mg/mL) were incubated with ca. 5 mCi of ^{3}HOH (Amersham, Arlington Heights, IL) in 1 M guanidinium chloride, 0.1 M NaH_2PO_4, pH 8.5 buffer for at least 18 h at room temperature. Free ^{3}HOH was removed from the protein solution with a PD-10 gel filtration column (Sigma), and exchange-out was thereby initiated. After recovery from the column, the protein solution was immediately transferred to prefilled Hamilton gastight syringes, containing buffer with the appropriate anesthetic concentration. Aliquots were delivered into 10% trichloroacetic acid at 2 °C at timed intervals for at least 6 h. The precipitated protein was vacuum-filtered through Whatman GF/B filters and washed with 8 mL of 1% trichloroacetic acid at 2 °C. ^{3}H retained by the protein was determined by liquid scintillation counting (*12, 31*). Protection factor ratios (PFR's) were determined by dividing the time required for a given hydrogen to exchange under the different conditions, for the last three to five hydrogens in common for the two conditions, and $\Delta\Delta G$ was determined using the equation $\Delta\Delta G = RT \ln(\text{PFR})$.

RESULTS

At all concentrations studied, dichloromethane destabilized myoglobin, according to all three of our methods of measuring stability. Sample traces with the three methods are shown in Figures 1−3. In Figure 1, differential scanning calorimetry traces show that the presence of 40 and 200 mM dichloromethane decreases the temperature (T_m) at which the peak in differential heat capacity (C_p) occurs for myoglobin unfolding. In Figure 2, circular dichroism spectroscopy measurements showed that myoglobin secondary structure is more susceptible to temperature-induced unfolding as the dichloromethane concentration is increased. Figure 3 shows that exchange of the last 30 tritiums out from labeled myoglobin is accelerated by these concentrations of dichloromethane at 45 °C. Thus, all three techniques indicate that dichloromethane reduces the stability of the folded state of myoglobin.

The T_m for myoglobin was 78.3 ± 0.1, 76.6 ± 0.3, and 63.2 ± 0.2 °C by calorimetry for 0, 40, and 200 mM dichloromethane (mean ± sem, $n = 2$ for each concentration), respectively, and 84.0 ± 0.6, 83.6 ± 0.3, and 70.5 ± 0.7 °C by circular dichroism measurements for those respective concentrations (mean ± sem, $n = 3$ for each). Thus, both methods indicate a concentration-dependent loss of folded stability induced by dichloromethane.

[1] Abbreviations: T_m, unfolding temperature; C_p, heat capacity; $\Delta\Delta G$, change in Gibbs energy change; ΔT_m, change in unfolding temperature; PFR, protection factor ratio; atm, atmosphere; sem, standard error of the mean.

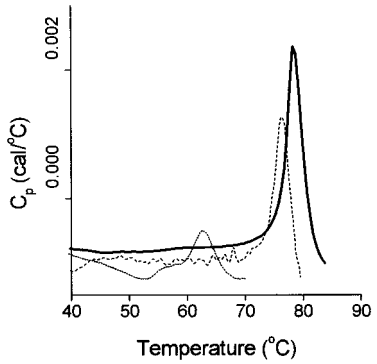

FIGURE 1: Differential heat capacity (C_p) versus temperature of myoglobin (2 mg/mL buffer = 150 mM NaCl, 20 mM KH_2PO_4, pH 7). Scan rate is 2 °C/min. Addition of 40 (dashed trace) and 200 (dotted trace) mM dichloromethane decreases the temperature (T_m) at which the peak in differential heat capacity (C_p) occurs for myoglobin unfolding, suggesting that dichloromethane has greater interaction with the unfolded state than with the folded state of myoglobin.

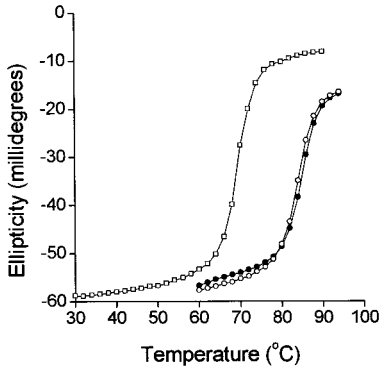

FIGURE 2: Ellipticity at 222 nm versus temperature of myoglobin (0.16 mg/mL, same buffer as Figure 1). Temperature was increased by 2 °C at a time, and samples were equilibrated for 2 min at each temperature. The bandwidth was 1 nm, and the measurement time was 10 s. The solid curves show the fits to the model equation given in the text. The results show that the α-helical content of myoglobin is more susceptible to temperature-induced unfolding with increasing concentrations of dichloromethane, again indicating that dichloromethane favors myoglobin unfolding. The conditions are control (solid circles), 40 mM dichloromethane (open circles), and 200 mM dichloromethane (open squares).

The enthalpy change, ΔH, for myoglobin unfolding calculated from the circular dichroism experiments is 125 ± 1, 124 ± 2, and 113 ± 2 kcal/mol (mean ± sem, $n = 3$ for each), for 0, 40, and 200 mM dichloromethane, respectively. Our measurement of ΔH in the absence of dichloromethane is in remarkable agreement with published calorimetry data under reversible conditions (*32*: 131 ± 5 kcal/mol). (Enthalpy changes measured in the calorimetry experiments presented here were smaller, probably due to irreversible aggregation of the protein at neutral pH; the concentration of protein for calorimetry experiments was higher than that for the circular dichroism experiments here, thus promoting aggregation.) Using these values for ΔH and the formula $\Delta\Delta G = \Delta H \cdot \Delta T_m / T_m$ (see Materials and Methods), the changes in the Gibbs energy change for unfolding ($\Delta\Delta G$) due to 40 and 200 mM dichloromethane are ca. −0.5 and −5 kcal/mol, respectively. This is in reasonable agreement with the $\Delta\Delta G$ values of −0.5 and −1.5 kcal/mol, respectively, obtained from the hydrogen exchange experiment using the formula $\Delta\Delta G = RT \ln(\text{PFR})$ (see Materials and Methods).

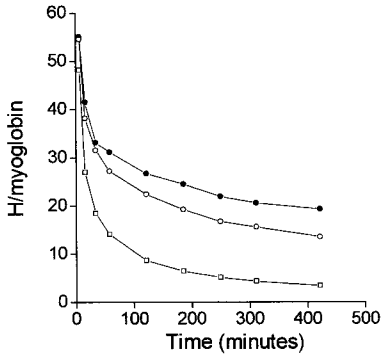

FIGURE 3: Hydrogen exchange of the last 30 tritiums out from labeled myoglobin is accelerated by dichloromethane, also indicating that dichloromethane destabilizes myoglobin. Again, the conditions are control (solid circles), 40 mM dichloromethane (open circles), and 200 mM dichloromethane (open squares). Exchange-out conditions were 150 mM NaCl, 20 mM KH_2PO_4, pH 7.0, and 45 °C.

It could be argued that these are higher concentration effects. We therefore performed calorimetry experiments at 15 mM dichloromethane, near clinical anesthetic concentrations. [At 37 °C, the solubility of dichloromethane in water is 12.356 g/L (*33*), and the vapor pressure of dichloromethane is 0.905 atm (*34*), so the anesthetic partial pressure of 0.03 atm corresponds to ca. 5 mM.] In this series, control T_m's were 78.5 ± 0.1 °C (mean ± sem, $n = 3$), whereas T_m's for samples with 15 mM dichloromethane were 77.8 ± 0.1 °C (mean ± sem, $n = 5$). These means are significantly different at the $p < 0.01$ level by a *t*-test.

Because the original crystallography experiments used sperm whale myoglobin, we performed differential scanning calorimetry experiments with recombinant sperm whale myoglobin. These studies showed that an intermediate concentration of dichloromethane (78 mM) reduced the T_m from 79.2 to 75.6 °C. Thus, species difference does not explain the discrepancy between our results, which were obtained predominantly with horse myoglobin, and what we predicted from the crystallography results of Nunes and Schoenborn (*4*). In calorimetry experiments done in the presence of 2.2 M ammonium sulfate, to mimic the crystallization conditions, dichloromethane also lowered the T_m. Also, to match the temperature of crystallography experiments, hydrogen exchange studies were performed at 20 °C, with 1 M guanidinium chloride added to the exchange-out buffer so measurements could be made within a reasonable time frame. Again, dichloromethane substantially accelerated hydrogen exchange from myoglobin, i.e., destabilized the folded protein state: 40 mM dichloromethane changed the ΔG for unfolding by about −0.4 kcal/mol, and 200 mM dichloromethane changed the ΔG by about −2 kcal/mol.

In contrast to the results with dichloromethane, 1 atm of xenon stabilized myoglobin. Sample differential scanning calorimetry experiments, showing that xenon increases the T_m for myoglobin, are shown in Figure 4. In a series of such experiments, control T_m's were 78.45 ± 0.06 °C (mean ± sem, $n = 4$), whereas T_m's for samples preexposed to 1 atm of xenon were 78.73 ± 0.03 °C (mean ± sem, $n = 3$). These means are significantly different at the $p < 0.02$ level by a *t*-test. This ΔT_m corresponds to a $\Delta\Delta G$ of $\Delta H \cdot \Delta T_m / T_m =$

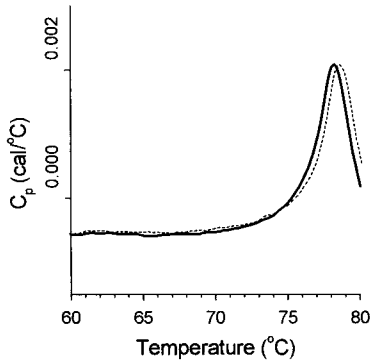

FIGURE 4: Differential heat capacity (C_p) vs temperature of myoglobin (same concentration, buffer, and scan rate as in Figure 1) in the absence (solid curve) and presence (dashed curve) of 1 atm of xenon, showing that xenon slightly stabilizes the folded state of myoglobin.

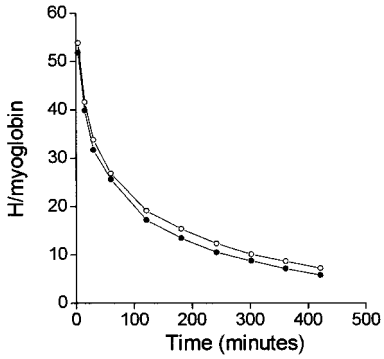

FIGURE 5: Hydrogen exchange experiment showing that hydrogen exchange is slightly slower in samples equilibrated with 1 atm of xenon (open circles) than in controls (closed circles), i.e., xenon slightly stabilizes myoglobin. Exchange-out conditions were 0.5 M guanidinium chloride, 0.1 M phosphate, pH 7.4, and 37 °C.

0.1 kcal/mol (using the value of ΔH given above). In agreement with these experiments, hydrogen exchange experiments (Figure 5) showed that xenon slightly stabilized myoglobin, by 0.1 kcal/mol.

DISCUSSION

Using three different techniques, we find that dichloromethane reduces the folded stability of myoglobin. This finding was contrary to the prediction from the crystallography results. It indicates that dichloromethane prefers interacting with the unfolded state of myoglobin over the interaction with the folded state illustrated by crystallography.

While X-ray crystallography has provided structural details not obtainable with other techniques, it comes with several caveats that must be acknowledged. First, crystallization of proteins may subtly change the structure from that in dilute aqueous solution (*35*, *36*), altering the features of the binding sites for small molecules such as inhaled anesthetics. Also, crystallization may suppress evidence of disorder; for example, solution-state nuclear magnetic resonance methods have demonstrated disorder in a region of the backbone of intestinal fatty-acid-binding apoprotein, but this disorder was not apparent from the X-ray crystal structure (*37*). For these reasons, solution studies are necessary to complement the information obtained from X-ray crystallography.

Furthermore, the conditions required for crystal formation favor protein−protein contacts, which stabilize the native folded state. Therefore, the folded protein state may be more stable under crystal conditions than under normal solution conditions. If ligand binding energies are not sufficient to overcome the additional stabilization of the folded state due to protein−protein interactions in the crystal, then the less folded state will not be populated, and binding to this state will not be revealed by crystallography. It is not even clear that the binding sites in the crystal are the strongest of those existing in solution. Sole reliance on X-ray crystallography may thus hamper understanding of anesthetic−protein interactions by providing misleading predictions of what protein conformations are stabilized by anesthetics.

Understanding which protein conformations are stabilized by ligands is of central importance for predicting functional effects. Ligands are thought to produce their effect on protein function by preferentially binding to conformations with particular functional characteristics and thereby shifting conformational equilibria to populate those conformations (*38*). Thus, identification of conformations stabilized by ligands is crucial to understanding effects of ligands on protein function. In particular, if solution experiments suggest preferential binding to a less folded state, then crystallography experiments that portray sites in the native state should be viewed cautiously, since the functionally relevant interactions may not be represented crystallographically.

The agreement among the three techniques demonstrated here bolsters our conclusion and allows us to rule out alternative explanations of the results. While it could be argued that the result with any one of these methods could be specious due to limitations of that particular method, the different methods complement each other. Protein stability (i.e., ΔG for unfolding) varies with temperature, so the observation that addition of ligand raises the T_m does not necessarily indicate that it also raises the ΔG for unfolding at ambient temperatures (cf. *39*). Furthermore, unfolded myoglobin is insoluble at neutral pH, so thermal melts are irreversible, and analysis with a reversible model is therefore not strictly valid. The T_m's measured by calorimetry and by circular dichroism spectroscopy were somewhat different; this could be due to differences in experimental factors such as protein concentration (that in the calorimetry experiments being higher, thereby accelerating irreversible aggregation and keeping protein unfolded at lower temperatures). The hydrogen exchange experiments, some of which were performed at 20 °C, do not suffer from these shortcomings of the thermal unfolding experiments. The consistent results in the hydrogen exchange experiments rule out the possibility that destabilization by dichloromethane is due solely to altering the rate of irreversible aggregation rather than also altering the unfolding equilibrium. On the other hand, the hydrogen exchange results could be explained by regional structural fluctuations (cf. *40*), rather than global effects on the folding equilibrium. Here again, results from the multiple approaches make this possibility unlikely. The thermal melting experiments strongly indicate that the anesthetic agents have an effect on global protein properties rather than merely regional structural fluctuations, reinforcing our interpretation that dichloromethane affects the myoglobin folding equilibrium.

The conditions of many of our experiments differed somewhat from those of the crystallography studies, and protein stability can be sensitive to conditions such as ionic

strength, temperature, and species. In an attempt to rule out the contribution of these factors to our findings, we performed some experiments where these conditions were similar to those of the crystallography studies: the use of sperm whale myoglobin, ambient temperature, and a high concentration of ammonium sulfate. Myoglobin is abundant in diving animals (*8*), but because the sperm whale is listed as an endangered species, most of our experiments used horse skeletal muscle myoglobin, which differs in 19 of the 153 amino acid residues (*41*) from sperm whale myoglobin, which was used by the crystallographers (*4*). In a calorimetry experiment performed with recombinant sperm whale myoglobin, however, dichloromethane lowered the T_m precisely as it did for horse myoglobin. Other experiments (calorimetry at 2.2 M ammonium sulfate and hydrogen exchange at 20 °C) confirm that dichloromethane reduces myoglobin stability even under the ionic and temperature conditions used in crystallography.

The X-ray crystals were prepared with dichloromethane at a partial pressure of 350 mmHg (*4*); this is the saturated vapor pressure of dichloromethane at 20 °C (*42*). Unfortunately, the relevant concentration of dichloromethane for myoglobin studies is unknown, because the binding constant of dichloromethane to myoglobin has not been reported, so we cannot rule out a subtle stabilizing effect of dichloromethane at very low concentration. Nevertheless, our use of a much broader range of dichloromethane concentrations, including one close to clinically relevant and a saturated one like the crystallography study, makes this possibility unlikely, and questionably relevant.

There are several possible explanations of why we found dichloromethane to destabilize myoglobin but xenon to stabilize it. As explained in the introduction, the net effect of a ligand on protein stability depends on a competition between binding to the folded state and binding to the unfolded state. It is possible that effects of xenon and dichloromethane differ because the number of binding sites of xenon to folded myoglobin is greater than that of dichloromethane. The binding constant of Xe has been reported as 1 atm at 20 °C (*43*). More recent X-ray diffraction of myoglobin crystals equilibrated with 7 atm of xenon has shown four xenon binding sites in myoglobin (*44*). Other binding sites for dichloromethane have not been reported, however, perhaps since it is about twice as large as xenon, dichloromethane does not fit into all the cavities in native-state myoglobin that bind xenon. Alternatively, the difference we observed could be explained by more nonspecific binding of dichloromethane to unfolded myoglobin and/or the higher aqueous concentration of dichloromethane than xenon in our experiments. We cannot yet distinguish among these possibilities.

The result with dichloromethane suggests that nonspecific interactions between this anesthetic agent and the unfolded protein actually have more effect on myoglobin than does binding at sites identified in the crystal structure, at least for determining the equilibrium between the folded and unfolded states. This demonstrates that interactions other than the specific ones shown by X-ray crystallography exist and can in fact dominate. Since X-ray crystallography may underreport functionally important interactions between weakly binding small molecules and proteins, other techniques must be applied to define the scope and consequences of such weak interactions. The present results demonstrate that inhaled anesthetic agents can alter protein stability in either direction at concentrations approaching those used clinically. Either stabilizing or destabilizing effects can alter protein function (*14*, and references cited therein), and identifying conformations stabilized by anesthetics is the key to understanding how anesthetics affect proteins.

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REFERENCES

1. Franks, N. P., and Lieb, W. R. (1994) *Nature 367*, 607−614.
2. Eckenhoff, R. G., and Johansson, J. S. (1997) *Pharmacol. Rev. 49*, 343−367.
3. Schoenborn, B. P., Watson, H. C., and Kendrew, J. C. (1965) *Nature 207*, 28−30.
4. Nunes, A. C., and Schoenborn, B. P. (1973) *Mol. Pharmacol. 9*, 835−839.
5. Sachsenheimer, W., Pai, E. F., Schulz, G. E., and Schirmer, R. H. (1977) *FEBS Lett. 79*, 310−312.
6. Franks, N. P., Jenkins, A., Conti, E., Lieb, W. R., and Brick, P. (1998) *Biophys. J. 75*, 2205−2211.
7. Bhattacharya, A. A., Curry, S., and Franks, N. P. (2000) *J. Biol. Chem. 275*, 38731−38738.
8. Kendrew, J. C., Bodo, G., Dintzis, H. M., Parrish, R. G., Wyckoff, H., and Phillips, D. C. (1958) *Nature 181*, 662−666.
9. Pace, C. N. (1990) *Trends Biochem. Sci. 15*, 14−17.
10. Schellman, J. A. (1975) *Biopolymers 14*, 999−1018.
11. Tanner, J. W., Liebman, P. A., and Eckenhoff, R. G. (1998) *Toxicol. Lett. 100−101*, 387−381.
12. Eckenhoff, R. G., and Tanner, J. W. (1998) *Biophys. J. 75*, 477−483.
13. Fink, A. L. (1986) *Cryobiology 23*, 28−37.
14. Tanner, J. W., Eckenhoff, R. G., and Liebman, P. A. (1999) *Biochim. Biophys. Acta 1430*, 46−56.
15. Johansson, J. S., Zou, H., and Tanner, J. W. (1999) *Anesthesiology 90*, 235−245.
16. Dubois, B. W., Cherian, S. F., and Evers, A. S. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 6478−6482.
17. Eckenhoff, R. G., and Shuman, H. (1993) *Anesthesiology 79*, 96−106.
18. Johansson, J. S., Eckenhoff, R. G., and Dutton, P. L. (1995) *Anesthesiology 83*, 316−324.
19. Bourne, W., and Stehle, R. L. (1923) *Can. Med. Assoc. J. 13*, 432−433.
20. Müller, J. (1925) *Arch. Exp. Pathol. Pharmakol. 109*, 276−294.
21. Grasset, J., and Gauthier, R. (1950) *Sem. Hop. 27*, 1280−1283.
22. Miller, K. W., Paton, D. M., and Smith, E. B. (1965) *Nature 206*, 574−577.
23. Cullen, S. C., and Gross, E. G. (1951) *Science 113*, 580−582.
24. Cullen, S. C., Eger, E. I., Cullen, B. F., and Gregory, P. (1969) *Anesthesiology 31*, 305−309.
25. Nakata, Y., Goto, T., and Morita, S. (1999) *Anesthesiology 90*, 406−410.
26. Lynch, C., III, Baum, J., and Tenbrinck, R. (2000) *Anesthesiology 92*, 865−870.
27. Trudell, J. R., Koblin, D. D., and Eger, E. I. (1998) *Anesth. Analg. (Baltimore) 87*, 411−418.
28. de Sousa, S. L. M., Dickinson, R., Lieb, W. R., and Franks, N. P. (2000) *Anesthesiology 92*, 1055−1066.
29. Funk, J., Pepling, M., Sage, G. W., Tunkel, J., Hueber, A., and Aronson, D. (1997) *Handbook of Physical Properties of Organic Chemicals*, Lewis Publishers, Boca Raton.

30. Becktel, W. J., and Schellman, J. A. (1987) *Biopolymers 26*, 1859−1877.
31. Eckenhoff, R. G. (1998) *Mol. Pharmacol. 54*, 610−615.
32. Kelly, L., and Holladay, L. A. (1990) *Biochemistry 29*, 5062−5069.
33. Horvath, A. L. (1982) *Halogenated Hydrocarbons: Solubility−Miscibility with Water*, p 484, Marcel Dekker, Inc., New York.
34. Boublík, T., Fried, V., and Hála, E. (1984) *The Vapour Pressures of Pure Substances*, p 41, Elsevier, Amsterdam.
35. Urbanova, M., Dukor, R. K., Pancoska, P., Gupta, V. P., and Keiderling, A. (1991) *Biochemistry 30*, 10479−10485.
36. Svergun, D. I., Barberato, C., Koch, M. H. J., Fetler, L., and Vachette, P. (1997) *Proteins: Struct., Funct., Genet. 27*, 110−117.
37. Hodsdon, M. E., and Cistola, D. P. (1997) *Biochemistry 36*, 1450−1460.
38. Freire, E. (1999) *Proc. Natl. Acad. Sci. U.S.A. 96*, 10118−10122.
39. del Pino, I. M. P., and Sanchez-Ruiz, J. M. (1995) *Biochemistry 34*, 8621−8630.
40. Mayo, S. L., and Baldwin, R. L. (1993) *Science 262*, 873−876.
41. Fasman, G. D. (1976) *Handbook of Biochemistry and Molecular Biology, 3rd edition, Proteins*, Vol. III, pp 437−440, CRC Press, Cleveland, OH.
42. Schoenborn, B. P. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 4195−4199.
43. Ewing, G. J., and Maestas, S. (1970) *J. Phys. Chem. 74*, 2341−2344.
44. Tilton, R. F., Kuntz, I. D., and Petsko, G. A. (1984) *Biochemistry 23*, 2849−2857.

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