Anesthetic Stabilization of Protein Intermediates: Myoglobin and Halothane[†]

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ABSTRACT: Halothane, an inhaled anesthetic, destabilizes the folded structure of myoglobin. To determine whether this is due to preferential interactions with less stable folded conformers of myoglobin versus the completely unfolded state, we used photoaffinity labeling, hydrogen exchange, fluorescence spectroscopy, and circular dichroism spectroscopy. Apomyoglobin was used as a model of a less stable conformer of myoglobin. Halothane destabilizes myoglobin and binds with low affinity and stoichiometry but stabilizes and binds with higher affinity to apomyoglobin. The same halothane concentration has no effect on cytochrome *c* stability. The apomyoglobin/halothane complex is favored at pH 6.5 as compared to pH 4.5 or pH 2.5. Halothane photoincorporates into several sites in apomyoglobin, some allosteric to the heme pocket. Guanidinium unfolding of myoglobin, monitored by CD spectroscopy, shows destabilization at less than 1.3 M Gdm but stabilization at greater than 1.3 M Gdm, consistent with the hypothesis that less stable conformers of myoglobin bind halothane preferentially. We suggest the structural feature underlying preferential binding to less stable conformers is an enlarged cavity volume distribution, since myoglobin has several intermediate-sized cavities, while cytochrome *c* is more well packed and has no cavities detected by GRASP. Specific binding to less stable intermediates may underlie anesthetic potentiation of protein activity.

Characterization of the interactions between inhaled anesthetics and proteins remains superficial despite decades of investigation (*1*). Most investigators have assumed a receptor−ligand sort of interaction for functionally important targets, but the impotent, small, and relatively featureless inhaled anesthetics raise the possibility that nonspecific interactions may be important. Nonspecific interactions with protein are often characterized as those that are dependent on exposed protein surface area, as opposed to any three-dimensional features of the protein. Thus, "nonspecific" refers to a preferential interaction between the ligand and the fully unfolded protein. However, we have previously reported that specific interactions, or those between the anesthetic ligand and protein sites formed by tertiary structure, correlate better with anesthetic pharmacodynamics than do nonspecific interactions (*2*). Apparent exceptions are troubling. For example, inhibition of the activity of firefly luciferase (FFL)[1] correlates well with anesthetic potency (*3*), yet the protein is consistently destabilized by anesthetics (*4*), suggesting nonspecific binding. In addition, regional dynamics of several membrane proteins are increased by anesthetics (*5*−*7*), also suggesting destabilization. Although these examples indeed might reflect nonspecific binding, we hypothesize that such destabilization is due to specific binding, but to a conformer of lower stability than the native state. For example, a normally inaccessible cavity in a protein structure becomes accessible, or gains favorable steric features, in a less stable conformer with greater conformational entropy. To test this hypothesis, we examined the interaction between halothane and a soluble protein with known, stable intermediates, myoglobin.

We have already reported that, like FFL, myoglobin is significantly destabilized by inhaled anesthetics (*8*), and others have used it as a model for binding interactions between inhaled anesthetic and protein (*9*). Removal of the heme from myoglobin produces a considerably less stable, but still largely native protein, apomyoglobin. Apomyoglobin can populate at least three different conformational ensembles, depending on the solvent conditions (*10*). At neutral pH, it exists as a nativelike structure, with about 55% α-helicity (compared to ∼70% in the holoprotein), but at pH 4.5 it exists as a less stable conformer with about 35% α-helicity (50% of native), and at pH 2.5, it is fully unfolded with essentially no α-helical character. By examining halothane photolabeling, fluorescence, circular dichroism spectroscopy, and hydrogen exchange of both myoglobin and apomyoglobin, we were able to determine the conformer with greatest affinity for halothane to suggest the mechanism of native state destabilization. Further, to relate this binding to an acquired structural feature on the intermediate, we digested and sequenced [^{14}C]halothane-labeled apoprotein.

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[1] Abbreviations: Gdm, guanidinium; FFL, firefly luciferase; $\Delta\Delta G$, change in free energy; CNBr, cyanogen bromide; CD, circular dichroism spectroscopy; PDB, Protein Data Bank; GABA, γ-aminobutyric acid; TCA, trichloroacetic acid.

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MATERIALS AND METHODS

Materials. Myoglobin, apomyoglobin (horse heart), and cytochrome *c* were obtained from Sigma (St. Louis, MO) and used without further purification. [^{14}C]Halothane (51 mCi/mmol) from DuPont-NEN (Boston, MA) was stored as a gas in the dark at room temperature. It was condensed and diluted immediately into buffer to a 7 mM solution immediately before use. ^{3}HOH was obtained from Amersham (Arlington Heights, IL) at 100 mCi/mmol. Sequenal grade guanidinium chloride was obtained from Pierce (Rockford, IL). All other chemicals were reagent grade or better and were obtained from Sigma.

Photolabeling. Proteins were dissolved at ~1−2 mg/mL in phosphate buffer, pH adjusted as indicated in the figure legends. All photolabeling buffers were first equilibrated with 100% argon. For the displacement experiments, increasing concentrations of unlabeled halothane were added to the protein solution in 2 mL, 0.5 mm path-length quartz cuvettes, the final addition being ~50 μM [^{14}C]halothane. This last addition eliminated any gas space from the cuvette, which was then tightly sealed with Teflon stoppers. Cuvettes were exposed to 254 nm light at 5 mm from an Oriel (Stratford, CT) Hg(Ar) pencil calibration lamp for 30 s, with constant mixing by enclosed microstir bars. Cuvette contents were precipitated with 2 mL of ice-cold 20% trichloroacetic acid (TCA), filtered through Whatman (Maidstone, U.K.) GF/B filters, and washed with 8 mL of ice-cold 2% TCA. Filters were equilibrated with 10 mL of fluor overnight, and retained dpm was determined in a Wallac (Perkin-Elmer, Wallac Inc., Gaithersburg, MD) β-counter. Each unlabeled halothane concentration was performed at least in triplicate, and the data are expressed as percent of control labeling.

To determine the labeling sites along the primary sequence of myoglobin, aliquots of protein were exposed to ~500 μM [^{14}C]halothane, with no added unlabeled halothane, exposed to UV as above for 60 s, and then washed by repeated filtration through Amicon 3 kDa centrifuge filters (Beverly, MA). These labeled proteins were lyophilized and resuspended in 70% formic acid containing 2:1 (by mass) CNBr:protein. Overnight incubation with argon and in the dark was followed by dilution with water and lyophilization. Lyophilized samples were dissolved in sample buffer and electrophoresed on tricine−polyacrylamide gels. Gels were dried and autoradiograms prepared. Some bands were eluted from the gels and further digested with V8 (endoprotease Glu-C), and the fragments were again separated on tricine gels, as indicated in the figure legends. Labeling of individual bands was determined from the ratio of reflectance density from the Commassie-stained gel and transmission density from the autoradiogram using Quantity-One (Bio-Rad, Hercules, CA) software. Bands for sequencing were electrophoretically transferred to PVDF membranes and then subjected to Edman degradation on a Applied Biosystems Model 473A sequencer (Foster City, CA). After identification of the fragment, larger samples were degraded with pre-HPLC fractions collected to determine release of radioactivity.

Hydrogen−Tritium Exchange. For the hydrogen−tritium exchange measurements, protein solutions (10 mg/mL) were incubated with ~5 mCi of ^{3}HOH in 1 M GdmCl and 0.1 M NaH_2PO_4, pH 8.5, buffer for at least 18 h at room temperature to ensure equilibration of all exchangeable hydrogens in the protein prior to initiation of exchange out. 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 (Reno, NV) gastight syringes, containing the appropriate anesthetic concentration and solvent conditions (see figure legends). Aliquots were precipitated with 2 mL of ice-cold 10% TCA at timed intervals over at least 6 h. The precipitated protein was vacuum filtered through Whatman GF/B filters and washed with 8 mL of ice-cold 2% TCA. ^{3}H retained by the protein was determined by liquid scintillation counting. 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 $\Delta\Delta G = RT \ln(\mathrm{PFR})$.

Fluorescence Spectroscopy. Halothane contains heavy atoms (Cl, Br) that can quench tryptophan fluorescence if bound in the immediate vicinity (<5 Å) (*11*). Thus, to determine if halothane can gain access to and exhibit selectivity for the tryptophan residues in myoglobin or apomyoglobin, increasing concentrations of halothane (from stock buffer solutions) were added to 4 mL fluorescence cuvettes containing ~2 μM protein and examined in a Shimadzu RF 5301 PC spectrofluorophotometer (Columbia, MD) using 295 nm excitation and emission scanning. Care was taken to eliminate all air from the cuvette with the last addition.

Circular Dichrosim Spectroscopy. Small samples (1.5 mL) of 0.0−3.2 M guanidinium chloride and 10 mM $NaPO_4$, pH 7.0, buffer were equilibrated with or without 10 mM halothane (final concentration) in Teflon-sealed microvials, and myoglobin (or cytochrome *c*) was added to a final concentration of about 7 μM. These samples were allowed to sit at 4 °C overnight, and then CD spectra were collected at 15 °C in an Aviv Model 62DS spectrometer (Lakewood, NJ).

RESULTS

As we have previously reported (*8*), myoglobin was photolabeled by halothane poorly, at low stoichiometry, and with no significant evidence of saturability (Figure 1). Apomyoglobin, on the other hand, demonstrated clear evidence of saturable binding (also Figure 1), with 50-fold greater stoichiometry than the holoprotein. We examined binding of both myoglobin and apomyoglobin under different pH conditions. Of these conditions, only the pH 6.5 apomyoglobin bound halothane significantly, indicating a requirement for structure for halothane binding (Figure 2). Guanidinium dramatically altered halothane photochemistry, so it was not possible to use this denaturant to populate less folded states of myoglobin or apomyoglobin for photolabeling studies.

Hydrogen-exchange studies in myoglobin demonstrate significant destabilization by halothane [and other inhaled anesthetics (*12*) and alcohols (*2*)] (Figure 3). In contrast, 7 mM halothane stabilized both the pH 4.5 and pH 6.5 form of apomyoglobin, as shown in Figure 4. However, the degree of stabilization of the pH 6.5 form ($\Delta\Delta G$ = 0.9 kcal/mol)

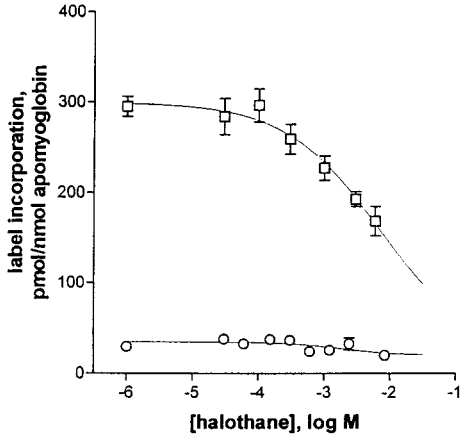

FIGURE 1: Photolabeling displacement studies show that the myoglobin (○) is labeled by halothane at low stoichiometry, without evidence of displacement by unlabeled halothane. Apomyoglobin is labeled at 10-fold higher stoichiometry with clear evidence of displacement. The apparent K_D for halothane is 5.1 ± 0.2 mM, and the Hill coefficient of −0.7 ± 0.1, assuming displacement to the level of myoglobin (nonlinear least squares was used to fit sigmoidal curves to the data, and values are means of three experiments ± SEM).

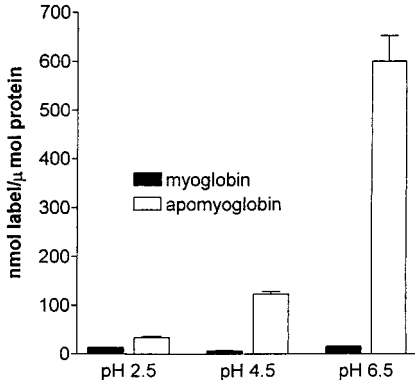

FIGURE 2: Label incorporation of myoglobin and apomyoglobin at different pH values. The pH 6.5 conformer of apomyoglobin is labeled to a significantly ($P < 0.001$) greater extent than either the pH 4.5 or pH 2.5 conformers. No significant pH effect can be demonstrated on photolabeling of myoglobin.

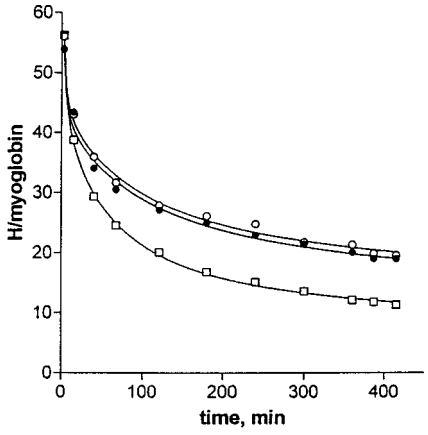

FIGURE 3: Amide hydrogen exchange out of myoglobin at 0 (open circles), 1 (closed circles), and 10 mM □ (open squares) halothane, demonstrating destabilization of the native structure [reprinted from *Biophys. J.* (1998) *75*, 477−483, with permission].

was significantly greater than that of the pH 4.5 form ($\Delta\Delta G$ = 0.4 kcal/mol), consistent with the enhanced binding by the pH 6.5 form as noted in the photolabeling studies. For

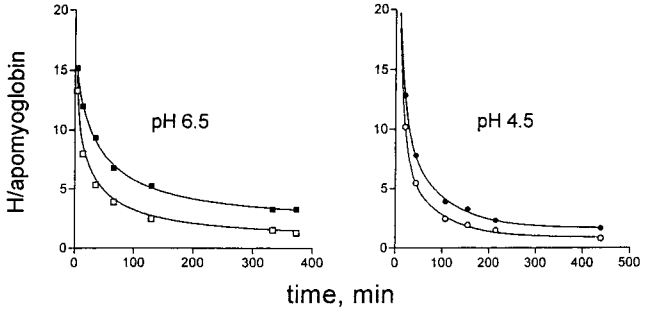

FIGURE 4: Amide hydrogen exchange out of apomyoglobin at two different pH values. As suggested by the results in Figure 2, the high-pH conformers of apomyoglobin (squares) were significantly better stabilized by 7 mM halothane (filled symbols) than the pH 4.5 conformers (circles), indicating preferential binding of halothane to apomyoglobin conformers with more structure. $\Delta\Delta G$ values are given in the text.

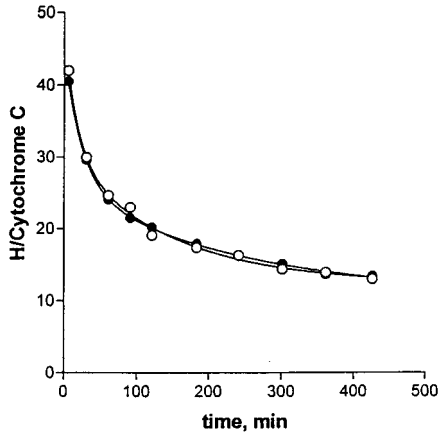

FIGURE 5: Amide hydrogen exchange out of cytochrome *c* with (filled circles) and without (open circles) the same concentration of halothane as in the above figures. There was no detectable effect of halothane in these experiments.

Table 1: Relative Label Incorporation into CNBr Fragments of Apomyoglobin

fragment		label incorporation
~MW	residues	OD/RD[a]
17	1−153	0.61
14.5	1−131	0.54
11	55−153	0.43
8	55−131	0.24
5	1−54	0.30

[a] OD/RD is the optical density of the autoradiogram normalized to the reflective density of the Commassie-stained gel.

comparison, we also examined a similarly sized heme-containing protein, cytochrome *c*. As shown in Figure 5, 7 mM halothane had no detectable effect on hydrogen-exchange rates in this heme-containing protein.

The pattern of CNBr fragment labeling of myoglobin and apomyoglobin was consistent with the filtration results for photolabeling—an approximately 10-fold higher label incorporation into apomyoglobin as compared to the holoprotein. Table 1 gives the relative label content of each major CNBr fragment from apomyoglobin. Although the small 23-residue C-terminal fragment was not recovered, the other two major CNBr fragments were labeled to a similar extent. From an analysis of partially digested material (Table 1), the C-terminal 23-residue fragment (helix H) must be labeled to a somewhat greater degree than the larger fragments. We

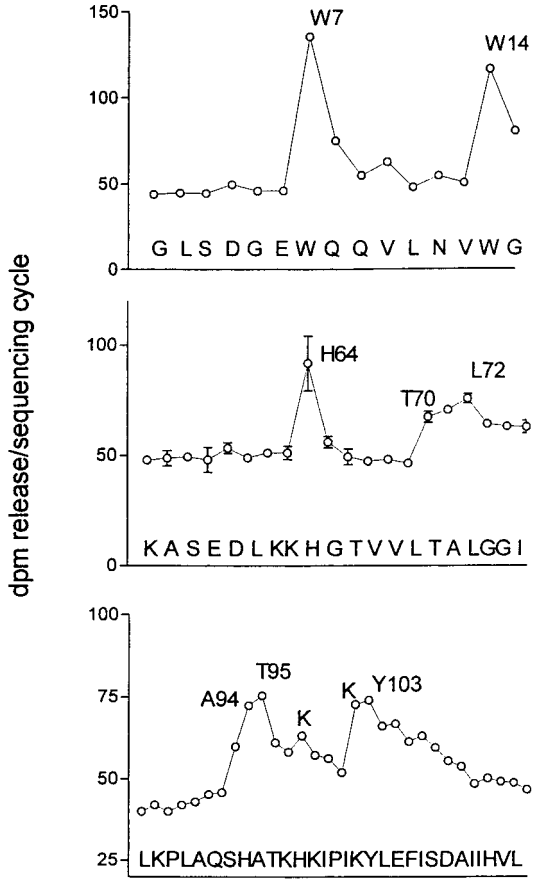

FIGURE 6: Radiosequencing of halothane-photolabeled apomyoglobin. Three different proteolytic fragments containing the majority of the label were sequenced, and the pattern of radioactivity release was noted. Several sites of incorporation were noted.

sequenced a total of 65 residues from three CNBr fragments for a total of 43% of apomyoglobin (we did not sequence the holoprotein, because of the low expected signal/noise). Radioactivity release was noted at a number of different residues from different locations in the molecule (Figure 6). Reconciling these locations along the primary structure with the three-dimensional structure of myoglobin (PDB no. 1YMB), it appears that halothane is interacting with at least three to four separate sites in the molecule, two of which could be considered to be in the immediate vicinity of the heme cavity (Figure 7).

Fluorescence Spectroscopy. The fluorescence measurements found that there was no significant effect of halothane on the already considerably quenched tryptophan fluorescence in the holoprotein or in the apoprotein at pH 2.5. Halothane did quench tryptophan fluorescence in the apoprotein at higher pH, with an EC_{50} of 6.6 ± 2.2 mM at pH 6.5 and an EC_{50} of 5.3 ± 1.0 mM at pH 4.5 (Figure 8). In both cases, only about 50% of the fluorescence signal could be quenched.

Circular Dichroism Spectroscopy. Figure 9 shows both representative spectra and the CD results at 222 nm with increasing guanidinium and with or without 10 mM halothane. Below 1.3 M guanidinium, halothane produces a small loss of helicity (−3 ± 0.8%) compared to control (no halothane), whereas above 1.3 M, halothane enhances helicity (+8.7 ± 2.7%). The crossover point is at about 1.3 M guanidinium, where helicity has been reduced by an average of 6% from native. The maximum stabilizing influence

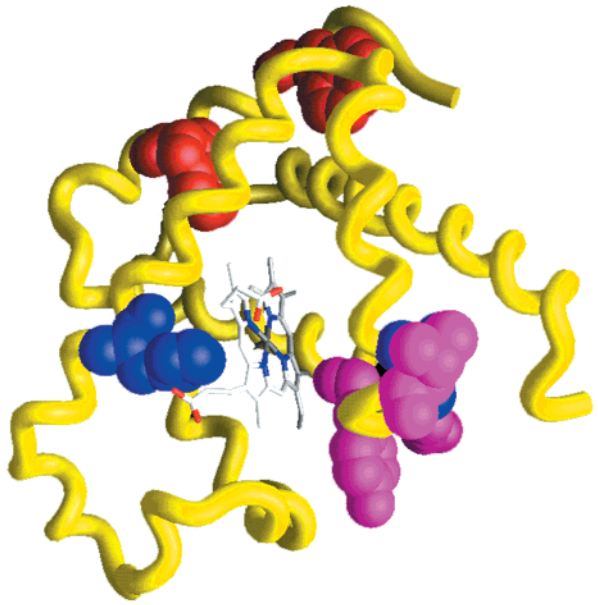

FIGURE 7: Ribbon structure of myoglobin (PDB no. 1YMB) with the labeled amino acids shown in space-filling representation (W7, W14, H64, T70, A71, L72, A94, T95, K102, Y103). There are at least three general regions of halothane incorporation, two of which are in the general region of the heme cavity.

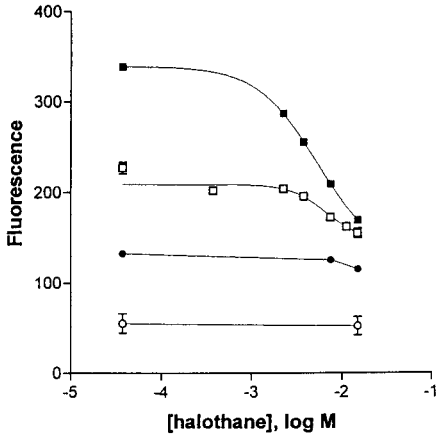

FIGURE 8: Fluorescence quenching by halothane of myoglobin and apomyoglobin at different pH values. The halothane IC_{50} value for pH 6.5 apomyoglobin (open squares) was 6.6 ± 2.2 mM, with a Hill coefficient of −2.2 ± 2.0; for pH 4.5 apomyoglobin (filled squares), IC_{50} was 5.3 ± 1.0 mM with a Hill coefficient of −1.3 ± 0.03 (nonlinear least squares was used to fit sigmoidal curves to the data, and values are means of three experiments ± SEM). The filled circles represent pH 2.5 apomyoglobin; the open circles, myoglobin.

(~18%) is observed at about the unfolding midpoint, 1.6 M guanidinium. In contrast to effects on myoglobin, halothane produced a negligible effect on the helicity of cytochrome *c* throughout the unfolding curve.

DISCUSSION

These data allow for a distinction between nonspecific binding and specific binding to intermediates as the basis for myoglobin destabilization by halothane. If myoglobin destabilization by halothane were solely due to nonspecific binding, the pH 2.5 form of apomyoglobin (and myoglobin) would have been a preferential binding conformer (because

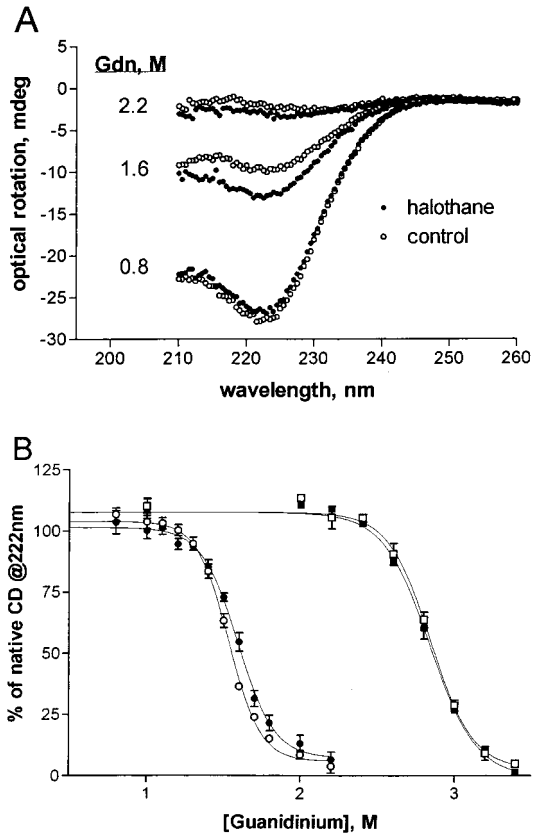

FIGURE 9: Guanidinium denaturation of myoglobin and cytochrome *c*. Panel A shows a typical circular dichroism spectroscopy experiment at three guanidinium concentrations, with (filled circles) and without (open circles) 10 mM halothane. Absorbance of both guanidinium and halothane precluded obtaining spectra below 210 nm. Panel B shows the CD signal at 222 nm for all guanidinium concentrations for both myoglobin (circles) and cytochrome *c* (squares). Halothane (filled symbols) produced a small loss of the 222 nm CD signal at <1.3 M guanidinium but enhanced this signal with [guanidinium] > 1.3 M. This concentration of guanidinium produces a helical content that corresponds approximately to that of apomyoglobin. In contrast, halothane had no detectable effect on the 222 nm CD signal of cytochrome *c* at any guanidinium concentration.

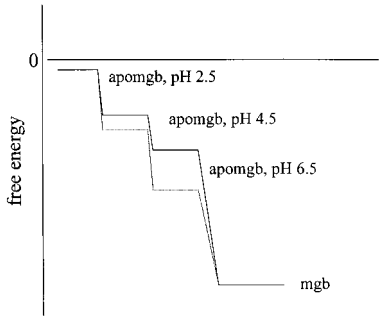

FIGURE 10: Hypothetical free energy diagram of the conformational equilibrium of myoglobin/apomyoglobin. The solid line depicts the native solution conditions and the dashed line that with halothane. Halothane binds preferentially to, and therefore stabilizes and populates, the conformers of intermediate stability, reducing the apparent stability of the entire ensemble. While this particular diagram assumes that apomyoglobin and its intermediates are on the folding pathway for myoglobin, it is likely to be valid even if the folding intermediates of myoglobin are different or are characterized as kinetic rather than equilibrium.

of larger solvent-exposed surface area), and unfolding of myoglobin would have been enhanced by halothane at all concentrations of guanidinium. Further, a similarly sized heme protein, cytochrome *c*, should have been similarly destabilized. However, our results indicated that more structured conformers of apomyoglobin bound halothane with higher affinity and stoichiometry, apomyoglobin was stabilized, and halothane had a biphasic effect on the unfolding of myoglobin by guanidinium. These new data strongly suggest that halothane destabilizes myoglobin through a preferential interaction with a conformer of intermediate stability instead of a nonspecific hydrophobic interaction with the completely unfolded protein (Figure 10). This is in accordance with previous studies of poly(L-lysine), which demonstrated that specific binding of halothane required tertiary structure (*13*).

This conclusion assumes that apomyoglobin and its intermediates adequately represent intermediates in the unfolding pathways for myoglobin itself. The CD experiments allowed a test of this assumption. At low guanidinium concentration (<1.3 M), halothane caused a decrease of the helical content of myoglobin, consistent with the destabilization observed by hydrogen exchange. However, the apomyoglobin experiments predict that halothane should stabilize myoglobin intermediates, thereby increasing helical content at guanidinium concentrations that populate these states. Accordingly, at guanidinium concentrations greater than 1.3 M we found that helical content was indeed enhanced by halothane. It is interesting to note that a 10−50% decrease in helicity (produced by guanidinium) is required to populate halothane-stabilized conformers, a similar difference between myoglobin and apomyoglobin (15−20%). Thermal unfolding should also populate these conformers, but we have been unable to show any stabilization (upshift) of myoglobin with halothane by differential scanning calorimetry (*12*). The probable explanation lies in the temperature dependence of the weak binding interaction and the fact that nonspecific hydrophobic interactions are enhanced at higher temperature. The ultimate effect of the anesthetic at body temperature, therefore, will depend on the binding affinity, the temperature dependence of binding, and the energetics of the protein conformational ensemble. Finally, it is not likely that the stabilization at >1.3 M guanidinium is due to a nonspecific helix stabilizing effect of halothane, like trifluoroethanol (*14*), because of the much lower concentration of cosolvent (halothane) in this case and the fact that we did not see a similar α-helical enhancement in cytochrome *c* with the same concentration of halothane and guanidinium.

Photolabeling clearly showed preferential labeling of the pH 6.5 conformer of apomyoglobin, consistent with the greater stabilization revealed by hydrogen−tritium exchange. On the other hand, fluorescence quenching by halothane was similar for both the pH 4.5 and pH 6.5 states of apomyoglobin, indicating relative preservation of the tryptophan-containing sites in these conformers. This suggests that the loss of halothane binding affinity in the pH 4.5 conformer as revealed by photolabeling and HX occurs largely through loss or changes in structure at the other sites indicated in Figure 7.

We propose that the acquired feature underlying preferential anesthetic binding in the myoglobin intermediates is an enlarged cavity volume. In support of this is our finding

that the general regions of apomyoglobin that are photolabeled correspond to lining residues of the larger cavities identified in the holoprotein structure by VOLBL (*15*) or GRASP (*16*). In the native structure, these cavities are too small (~90 Å^3) to accommodate halothane (~130 Å^3), but it is probable that normal conformational dynamics (*17*) produce a large enough distribution of cavity volume to overlap the volume of halothane. Thus, providing a sufficiently high concentration of ligand to enter and stabilize these transiently enlarged cavities will trap some protein molecules in the more dynamic conformation, resulting in apparent destabilization of the overall population. In the case of apomyoglobin, the highly dynamic dominant population of molecules is expected to have a larger cavity volume distribution than myoglobin and therefore be stabilized by ligands of the appropriate size (*18*). The above predicts, of course, that myoglobin should be stabilized by ligands of smaller size, which we have confirmed at least in the case of xenon (*19*). In very well packed proteins such as cytochrome *c* (no cavities detected by GRASP), where creation of large enough cavities would require more complete unfolding, less destabilization will be observed because of the lower frequency of such large global events and the weak binding energetics of the anesthetic.

A portion of the binding energy of the inhaled anesthetics is used to alter the protein conformation to the more dynamic state (ΔG_{conf}), so relatively high concentrations of the anesthetic are required to significantly populate the intermediate states. For this reason, and because we found that stabilizing influences correlated better with anesthetic potency than destabilizing ones (*2*), we suggest that destabilizing influences, or those caused by preferential binding to less stable intermediates, are not likely to underlie important actions of these drugs on protein targets in the central nervous system. On the other hand, if intermediates are first stabilized through higher affinity interactions with endogenous ligands, then anesthetics could contribute by further stabilizing these conformers and be experimentally evident as an increased apparent affinity of the endogenous ligand. Interestingly, previous studies have documented that anesthetics produce an increase in the apparent affinity of agonists (GABA, glycine, acetylcholine) for the ligand-gated ion channel family (*20*−*22*), as well as of the nucleotide in the case of FFL (*23*, *24*). Thus, both stabilizing and destabilizing influences on isolated proteins may reflect important interactions, depending on the energetic and steric relationship between the native (reference) state and the conformer of interest.

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