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A calorimetric study on the binding of six general anesthetics to the hydrophobic core of a model protein

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Abstract

The thermodynamic parameters underlying the binding of six volatile general anesthetics to the hydrophobic core of the four-α-helix bundle $(A\alpha_2\text{-}L38M)_2$ are determined using isothermal titration calorimetry. Chloroform, bromoform, trichloroethylene, benzene, desflurane and fluroxene are shown to bind to the four-α-helix bundle with dissociation constants of 880±10, 90±5, 200±10, 900±30, 220±10 and 790±40 μM, respectively. The measured dissociation constants for the binding of the six general anesthetics to the four-α-helix bundle $(A\alpha_2\text{-}L38M)_2$ correlate with their human or animal EC_{50} values. The negative enthalpy changes indicate that favorable polar interactions are achieved between bound anesthetic and the adjacent amino acid side chains. Because of its small size and the ability to bind a variety of general anesthetics, the four-α-helix bundle $(A\alpha_2\text{-}L38M)_2$ represents an attractive system for structural studies on anesthetic–protein complexes.
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1. Introduction

The mechanisms of action of volatile general anesthetics remain obscure because of the relatively weak interaction potential that characterizes these widely used clinical compounds. The low affinity with which volatile general anesthetics bind to macromolecules has made conclusive identification of the in vivo targets by direct binding studies a challenge [1]. Current consensus favors direct interactions with ligand-gated ion channels in the central nervous system, such as the γ-aminobutyric acid type A receptor, as playing an important role in the anesthetic state [2–4]. Although a high-resolution structure of the γ-aminobutyric acid type A receptor is not currently available, a recent cryoelectron microscopy study at 4 Å resolution on the homologous nicotinic acetylcholine receptor from *Torpedo marmorata* indicates that the transmembrane domain of each subunit consist of a four-α-helix bundle [5]. Studies on volatile general anesthetic binding to isolated four-α-helix bundles are therefore being carried out [6–9] because there is evidence from photoaffinity labeling experiments that general anesthetics interact directly with the transmembrane domains of membrane proteins [10–12]. Because of the relatively small size of this four-α-helix bundle motif (124 amino acids), it is possible to carry out a variety of biophysical studies that are not feasible in the case of intact ligand-gated ion channels, even if it was possible to overcome the current technical obstacles to obtaining a sufficient amount of purified material.

In the current manuscript, the binding of six additional volatile general anesthetics to the hydrophobic core

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of the four-α-helix bundle $(A\alpha_2\text{-}L38M)_2$ is examined using isothermal titration calorimetry. The current work builds on an earlier study using isothermal titration calorimetry [13] that characterized the binding of halothane, isoflurane, enflurane and sevoflurane to the same protein. The binding of chloroform to the four-α-helix bundle $(A\alpha_2\text{-}L38M)_2$ as determined using isothermal titration calorimetry is first investigated in order to further calibrate the validity of the results against prior fluorescence quenching data examining this interaction [14]. The thermodynamic characterization of the binding of bromoform, trichloroethylene, benzene, desflurane and fluroxene to the four-α-helix bundle $(A\alpha_2\text{-}L38M)_2$ is then presented.

2. Experimental

2.1. Materials

9-Fluorenylmethoxycarbonyl (Fmoc) amino acids and Fmoc 2,4-dimethoxybenzhydrylamide resin were purchased from Perkin Elmer (Foster City, CA). Chloroform (99.8%), bromoform (99+%), trichloroethylene (99.5+%) and benzene (99.8%) were purchased from Aldrich Chemical (Milwaukee, WI). Desflurane (1,2,2,2-tetrafluoroethyl difluoromethyl ether; Fig. 1) was purchased from Baxter (Deerfield, IL), and fluroxene (2,2,2-trifluoroethyl vinyl ether) from Ohio Medical Products (Madison, WI). All other chemicals were of reagent grade.

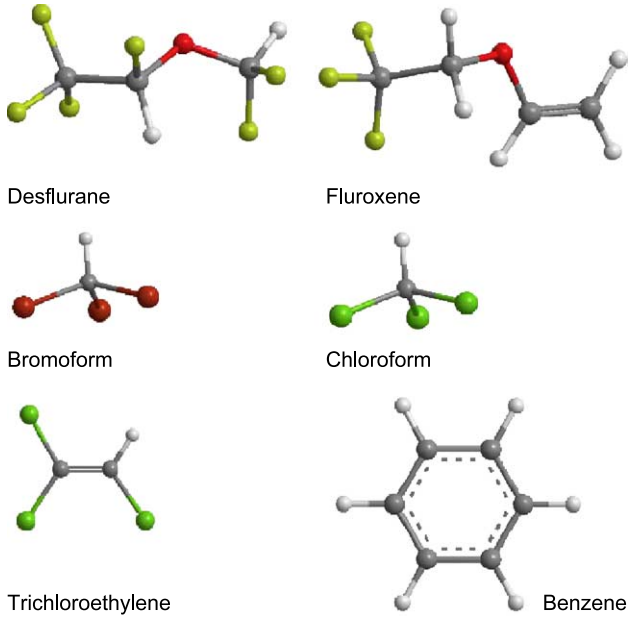

Fig. 1. Chemical structures of desflurane, fluroxene, bromoform, chloroform, trichloroethylene and benzene. Desflurane is represented as a single optical isomer, although a 50:50 mixture of the D- and L-enantiomers was used. Carbon is gray, hydrogen is white, oxygen is red, chlorine is green, bromine is maroon, and fluorine is lime.

2.2. Peptide synthesis and preparation

The peptide $A\alpha_2$-L38M [7] was assembled as a C-terminus carboxyamide on 0.25 mM scale using Fmoc amino acids and Fmoc 2,4-dimethoxybenzhydrylamide resin on an Applied Biosystems Model 433A (Perkin Elmer) solid-phase peptide synthesizer. Crude peptides were purified to homogeneity using reversed-phase C_{18} HPLC with aqueous–acetonitrile gradients containing 0.1% (vol/vol) 2,2,2-trifluoroacetic acid. Laser desorption mass spectrometry confirmed the peptide identity.

2.3. Isothermal titration calorimetry

Isothermal titration calorimetry was performed using a MicroCal VP-ITC titration microcalorimeter (Northampton, MA) at 20 °C for five of the anesthetics. In the case of desflurane, isothermal titration calorimetry was carried out at 10 °C, since the boiling point of this anesthetic is 22.8 °C at 1 atm [15]. The four-α-helix bundle $(A\alpha_2\text{-}L38M)_2$ at a concentration of 123 μM in 130 mM NaCl, pH 7.0, was placed in the 1.4-ml calorimeter cell, and anesthetic (5 mM in 130 mM NaCl, pH 7.0) was added sequentially in 10-μl aliquots (for a total of 29 injections) at 5-min intervals. The heat of reaction per injection (microcalories per second) was determined by integration of the peak areas using the Origin Version 5.0 software (1998). This software provides the best fit values for the heat of binding (ΔH°), the stoichiometry of binding (n) and the association constant (K_a) from plots of the heat evolved per mole of anesthetic injected vs. the anesthetic/$(A\alpha_2\text{-}L38M)_2$ molar ratio [16]. The heats of dilution were determined in parallel experiments by injecting either 130 mM NaCl, pH 7.0 into a 123 μM four-α-helix bundle $(A\alpha_2\text{-}L38M)_2$ solution or 5 mM anesthetic (in 130 mM NaCl, pH 7.0) into the 130 mM NaCl, pH 7.0 solution. These heats of dilution are subtracted from the corresponding four-α-helix bundle-anesthetic binding experiments prior to curve fitting.

The overall shape of the titration curve depends upon the c value $([(A\alpha_2\text{-}L38M)_2]/K_d)$ [16] and is rectangular for high c values (>500) and flat for low c values (<0.1). Earlier work using tryptophan fluorescence quenching indicates that chloroform binds to the four-α-helix bundle $(A\alpha_2\text{-}L38M)_2$ with a K_d of 1.4±0.2 mM [14]. To achieve a c value in the ideal range for isothermal titration calorimetry (5–50) would therefore require prohibitively high concentrations of protein (on the order of 7–70 mM). The four-α-helix bundle concentration used was 123 μM (c=0.6), resulting in a shallow titration curve for chloroform. Because the hydrophobic core of the four-α-helix bundle $(A\alpha_2\text{-}L38M)_2$ contains two identical binding sites for the anesthetics [13], n was set as 1.0 so that deconvolution of the resulting isotherms only required the K_a and ΔH° values to be minimized. Allowing all three variables to float simultaneously during the curve-fitting procedure may be

associated with more variable results because of the potential for multiple minima [16].

3. Results

3.1. Binding of the volatile anesthetic chloroform to the hydrophobic core of the four-α-helix bundle ($A\alpha_2$-L38M)$_2$

Fig. 2A shows a calorimetric titration at pH 7.0 of the four-α-helix bundle ($A\alpha_2$-L38M)$_2$ with chloroform. In the upper panel in Fig. 2A, each peak in the binding isotherm reflects a single injection of chloroform. The negative deflections from the baseline upon each addition of chloroform indicate that heat was evolved (an exothermic process). The enthalpy change associated with each injection of chloroform was plotted vs. the chloroform/($A\alpha_2$-L38M)$_2$ molar ratio (lower panel, Fig. 2A), and the ΔH°, K_d, the free energy change associated with binding (ΔG°) and the change in entropy associated with binding (ΔS°) were determined from the plots. The K_d value of 880±10 μM is quite comparable to the value of 1.4±0.2

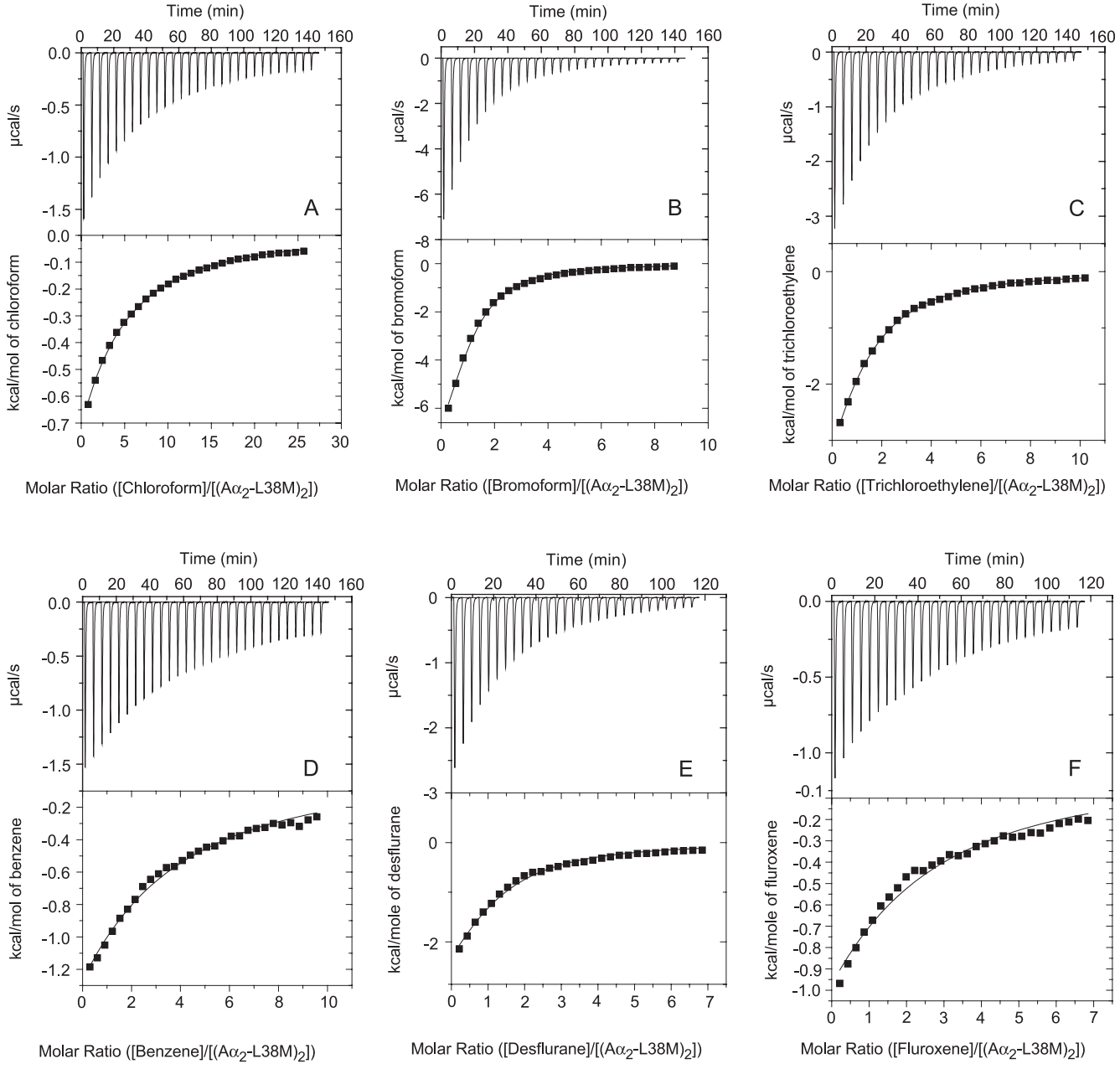

Fig. 2. Titration of the four-α-helix bundle ($A\alpha_2$-L38M)$_2$ with (A) chloroform, (B) bromoform, (C) trichloroethylene, (D) benzene, (E) desflurane and (F) fluroxene, showing the calorimetric response as successive injections of ligand are added to the reaction cell. In each case, the lower panel depicts the binding isotherm of the calorimetric titration shown in the upper panel. The continuous lines in the lower panels represent the least squares fit of the data to a single-site binding model.

mM obtained using tryptophan fluorescence quenching [14] supporting the validity of the results. Table 1 contains the remaining thermodynamic parameters underlying chloroform binding to the four-α-helix bundle $(A\alpha_2\text{-L38M})_2$.

3.2. Binding of the general anesthetics bromoform, trichloroethylene, benzene, desflurane and fluroxene to the hydrophobic core of the four-α-helix bundle $(A\alpha_2\text{-L38M})_2$

Fig. 2B–F shows calorimetric titrations at pH 7.0 of the four-α-helix bundle $(A\alpha_2\text{-L38M})_2$ with bromoform, trichloroethylene, benzene, desflurane and fluroxene. Each peak in the binding isotherms (upper panels, Fig. 2B–F) are the result of a single injection of the different anesthetics. The negative deflections from the baseline on addition of anesthetic in each case indicate that heat was evolved. The enthalpy change associated with each injection of anesthetic was plotted vs. the anesthetic/$(A\alpha_2\text{-L38M})_2$ molar ratio (lower panels, Fig. 2B–F), and the ΔH°, K_d, ΔG° and ΔS° were determined from the plots. The K_d values for bromoform, trichloroethylene, benzene, desflurane and fluroxene binding to the four-α-helix bundle $(A\alpha_2\text{-L38M})_2$ were 90±5, 200±10, 900±30, 220±10 and 790±40 μM, respectively. Table 1 shows the ΔH°, ΔG° and ΔS° values underlying the binding of these five general anesthetics to the four-α-helix bundle $(A\alpha_2\text{-L38M})_2$.

3.3. The Meyer–Overton rule is obeyed by the four-α-helix bundle $(A\alpha_2\text{-L38M})_2$

One of the fundamental tests of whether a reductionist system adequately models the actual in vivo sites of general anesthetic action is its ability to follow the Meyer–Overton rule [17]. A Meyer–Overton plot correlates the binding affinities of anesthetics to a given macromolecule (or their EC_{50} values for altering the activity of a protein) with their potencies for causing the anesthetic state in intact organisms. Fig. 3 shows a plot of the experimentally determined dissociation constants for the six anesthetics included in the current study (along with previously measured values for halothane, chloroform, isoflurane, sevoflurane and enflurane binding to the hydrophobic core of the four-α-helix bundle $(A\alpha_2\text{-L38M})_2$) vs. human or whole animal potency (EC_{50})

Table 1
Dissociation constants and thermodynamic data for binding of volatile general anesthetics to the four-α-helix bundle $(A\alpha_2\text{-L38M})_2$

Anesthetic	K_d (μM)	ΔG° (kcal/mol)	ΔH° (kcal/mol)	ΔS° (eu)
Chloroform	880±10	−4.1±0.1	−7.4±0.1	−11.2
Bromoform	90±5	−5.4±0.1	−10.5±0.1	−17.2
Trichloroethylene	200±10	−5.0±0.1	−8.2±0.1	−11.2
Benzene	900±30	−4.1±0.1	−10.6±0.2	−22.3
Desflurane	220±10	−4.9±0.1	−5.1±0.1	−1.3
Fluroxene	790±40	−4.2±0.1	−5.5±0.2	−5.3

The entropy unit (eu) is cal/mol K.

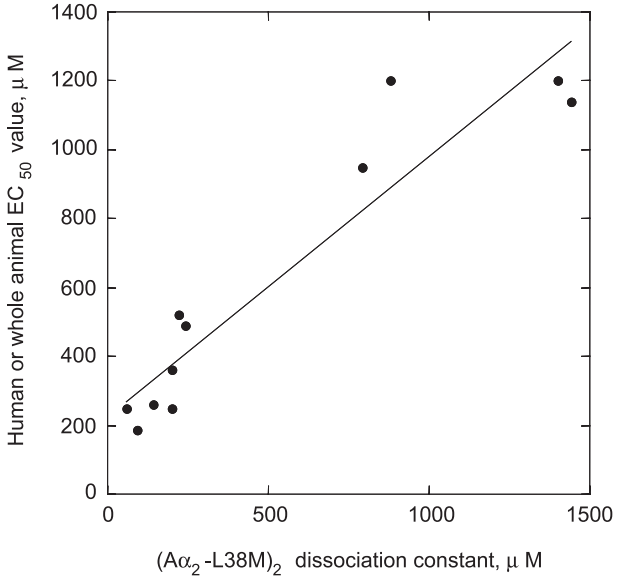

Fig. 3. Meyer–Overton plot of the human or whole animal potency data vs. the experimental dissociation constant for binding to the four-α-helix bundle $(A\alpha_2\text{-L38M})_2$. The halothane clinical EC_{50} value in man is 250 μM [2], the chloroform EC_{50} value in dogs is 1.2±0.2 mM [27], and the EC_{50} values in humans for isoflurane, sevoflurane and enflurane are 260, 260, and 490 μM, respectively [3]. The anesthetic EC_{50} value for bromoform in tadpoles is 185±3 μM [23], for trichloroethylene in rats, it is 0.36 mM [24], and for benzene in rats, it is 1.1 mM [22]. The anesthetic EC_{50} values for desflurane and fluroxene in humans are 0.52 and 0.95 mM, respectively [25]. Two data points are for chloroform, using the current K_d value and that of 1.4±0.2 mM determined using fluorescence spectroscopy as reported earlier [14]. There are also two data points for halothane, indicating the K_d values determined using fluorescence quenching [7] and isothermal titration calorimetry [13]. Note that the isoflurane and sevoflurane data points superimpose. The least squares linear regression correlation coefficient is 0.941.

data. It is apparent that the binding site in the hydrophobic core of the four-α-helix bundle $(A\alpha_2\text{-L38M})_2$ behaves in accord with the Meyer–Overton rule, suggesting that it represents a reasonable structural model of the actual central nervous system target sites.

4. Discussion

The current study shows that the hydrophobic core of the four-α-helix bundle $(A\alpha_2\text{-L38M})_2$ is able to bind six additional volatile general anesthetics as determined using isothermal titration calorimetry. The affinities with which chloroform, bromoform, trichloroethylene, benzene, desflurane and fluroxene bind to $(A\alpha_2\text{-L38M})_2$ are shown to correlate with their EC_{50} values for maintaining the anesthetic state, either in man or in intact animals. This builds on an earlier study that showed that halothane, isoflurane, enflurane and sevoflurane bound to the four-α-helix bundle $(A\alpha_2\text{-L38M})_2$ with dissociation constants that mirrored their respective clinical EC_{50} values in man [13].

To date, there have been few direct studies using equilibrium approaches to define the energetics of chloroform binding to proteins. Using a competition approach with halothane, chloroform binding to bovine serum albumin yielded a K_i=1.3±0.1 mM [18]. The average affinity of the two detectable binding sites for chloroform on bovine serum albumin was 2.7±0.2 mM as reported by intrinsic tryptophan fluorescence quenching [19]. Finally, using W15 fluorescence quenching, chloroform was found to bind to the hydrophobic core of $(A\alpha_2\text{-}L38M)_2$ with a K_d of 1.4±0.2 mM [14]. The K_d for chloroform binding of 880±10 μM determined using isothermal titration calorimetry in the current study is therefore in reasonable agreement with the latter value obtained using a spectroscopic approach.

Benzene was shown to bind to the L99A cavity created in the interior of phage T4 lysozyme with a K_d=175±40 μM using isothermal titration calorimetry [20] and a K_d of 0.40 mM using a thermal unfolding assay as monitored by circular dichroism spectroscopy [21]. The current results indicate that benzene binds to the four-α-helix bundle $(A\alpha_2\text{-}L38M)_2$ with a K_d=900±30 μM. These values are all in reasonable agreement with the EC_{50} value in rats of 1.1 mM [22].

No reports that directly address bromoform, trichloroethylene, desflurane, or fluroxene binding to proteins are currently available in the literature. The anesthetic EC_{50} value for bromoform in tadpoles is 185±3 μM [23], and for trichloroethylene in rats, it is 0.36 mM [24]. The anesthetic EC_{50} values for desflurane and fluroxene in humans are 0.52 and 0.95 mM, respectively [25]. The finding that bromoform, trichloroethylene, desflurane and fluroxene bind to the four-α-helix bundle $(A\alpha_2\text{-}L38M)_2$ with K_d values of 90±5, 200±10, 220±10 and 790±40 μM, respectively, therefore indicates that the affinities in each case approximate the whole animal or human EC_{50} values.

The negative enthalpy changes (ΔH°) associated with the binding of chloroform, bromoform, trichloroethylene, benzene, desflurane and fluroxene to the hydrophobic core of the four-α-helix bundle $(A\alpha_2\text{-}L38M)_2$ indicate that favorable polar interactions are achieved between bound anesthetic and the adjacent amino acid side chains. For each volatile general anesthetic examined in the current study, the ΔH° value for binding to the hydrophobic core of the four-α-helix bundle $(A\alpha_2\text{-}L38M)_2$ was more negative than the free energy change associated with binding (ΔG°). The entropy changes associated with the binding (ΔS°) of each of the six volatile general anesthetics, therefore, have negative values and as such act to oppose complex formation. This net entropic loss reflects relatively high-affinity binding of anesthetic to the hydrophobic core of the four-α-helix bundle $(A\alpha_2\text{-}L38M)_2$, which results in a decrease in the translational and rotational degrees of freedom of both the anesthetic and the protein side chains at the binding site [26].

5. Conclusions

Taken together with the earlier study [13], the current results indicate that this relatively small protein is able to bind a total of 10 volatile general anesthetics with affinities that approximate their respective EC_{50} values for the anesthetic state, either in man or in intact animals. This suggests that the four-α-helix bundle $(A\alpha_2\text{-}L38M)_2$ represents a good model for the in vivo central nervous system sites of general anesthetic action. The four-α-helix bundle $(A\alpha_2\text{-}L38M)_2$ is therefore an attractive system for high-resolution structural studies on the anesthetic complexes using X-ray crystallography and nuclear magnetic resonance spectroscopy. Such studies will provide much needed insight at the molecular level into how anesthetics reversibly alter protein function.

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