

Weak Polar Interactions Confer Albumin Binding Site Selectivity for Haloether Anesthetics

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Background: Enflurane and isoflurane are structural isomers with different anesthetic potencies and side effects. It is not clear whether these differences are produced by differing occupancy of common protein binding sites or by occupancy of different sites, but the very similar molecular properties make the latter possibility unlikely. In this study, the authors examined binding site selectivity of these anesthetics in human serum albumin (HSA).

Methods: Binding of isoflurane and enflurane with HSA was determined with isothermal titration calorimetry. Competition with known ligands (propofol) allowed localization of binding sites within the HSA molecule. Molecular properties of isoflurane and enflurane were calculated.

Results: Isoflurane binds HSA with higher affinity but smaller total enthalpy than enflurane. Enthalpogram analysis suggested that isoflurane bound a single site, whereas enflurane bound two. Competition experiments indicated that enflurane and isoflurane share one binding site, which also binds propofol. The additional enflurane site binds propofol but not isoflurane. Increased salt concentration decreased the affinity for isoflurane but not for enflurane. The dipole moment of isoflurane is higher than that of enflurane, and the isoflurane binding site is more polar.

Conclusion: These data indicate two binding sites of different character for the haloether anesthetics on HSA. One site is more polar and prefers isoflurane, presumably because of its larger dipole. The second site prefers the less polar enflurane. Therefore, weak polar interactions confer considerable selectivity, and differences in drug action may arise from occupancy of different protein sites.

It is now well accepted that direct interactions between anesthetic and protein might contribute to anesthesia and that internal protein cavities are favored anesthetic binding sites.^{1–4} The strong relation between potency and hydrophobicity suggests that these internal cavities are hydrophobic and of sufficient volume to accommodate a range of molecular sizes. However, because the molecular volumes of the inhaled anesthetics are similar, it is not clear whether differences in drug action are due to differential occupancy and effects at common binding sites or different binding sites altogether. If the latter is true, other interactions must provide the basis for binding selectivity. However, these volatile compounds have no formal charge and minimal dipole or hydrogen bonding potential.

Enflurane (2-chloro-1,1,2-trifluoroethyl difluoromethyl ether) and isoflurane (1-chloro-2,2,2-trifluoroethyl difluoromethyl ether) are structural isomers, having the same atomic composition and essentially the same molecular volume but different atomic and electrostatic potential distribution (fig. 1). Their physiologic effects are also different. For example, enflurane is 50% less potent than isoflurane and has different effects on the circulatory and respiratory systems. Enflurane has also been associated with seizure-like electroencephalographic activity in some situations and is metabolized to a greater extent than isoflurane. If different protein binding sites underlie these different effects, features other than volume must provide for the selectivity.

In this study, we compared isoflurane and enflurane binding energetics with human serum albumin (HSA) using isothermal titration calorimetry (ITC) and correlated the results with molecular properties of the two molecules. We hypothesize that selectivity for binding sites will be demonstrated and that the basis for this is dipole moment.

Materials and Methods

Human serum albumin (essentially fatty acid free) was purchased from Sigma Chemical Company (St. Louis, MO) and used without further purification. Isoflurane and enflurane were obtained from Halocarbon Laboratories (Liberty Corner, NJ). All other chemicals were reagent grade or better and were obtained from Sigma.

Isothermal Titration Calorimetry

Isothermal titration calorimetry can measure the full thermodynamic profile of a bimolecular interaction (binding) without modification of the ligand or the protein. The method consists of an ultrasensitive thermometer to measure the heat changes that occur when ligand and protein are mixed, and by fitting the heat signal of multiple, systematic injections of ligand into protein solutions, various binding models can be used to derive the underlying thermodynamics (enthalpy and entropy changes), including the association constant (K_A) and stoichiometry (n). We and others have demonstrated good agreement with data derived from other techniques.^{5,6}

Briefly, titrations were performed at 20°C using a Microcal, Inc. VP ITC (Northampton, MA). The sample cell contained 0.21 mM HSA, and the reference cell contained water. Ligand, 15 μ l (injector stock concentrations of 12 mM for isoflurane and 10 mM for enflurane), was injected

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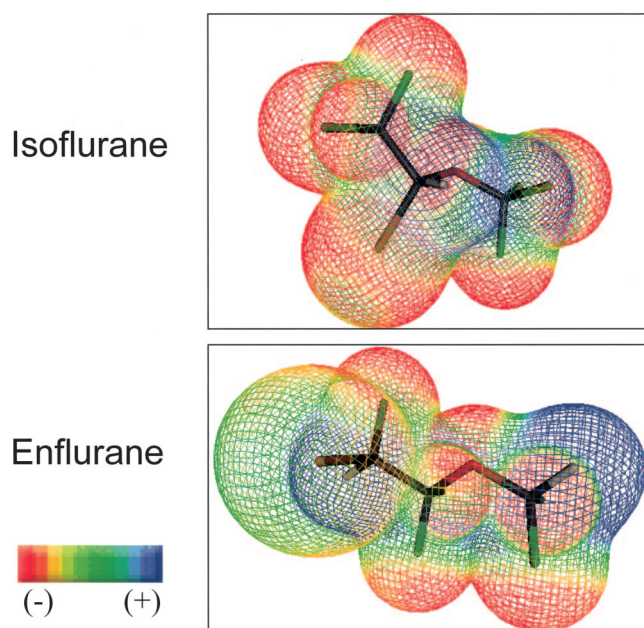


Fig. 1. Electrostatic potential maps of isoflurane and enflurane. Red is negative and blue is positively charged molecular surface. Note the different position of the largest contributor to the electrostatic potentials, the chlorine. These maps were obtained from <http://www.ps.toyaku.ac.jp/dobashi> (accessed February 10, 2004). Enflurane: 2-chloro-1,1,2-trifluoroethyl difluoromethyl ether; isoflurane: 1-chloro-2,2,2-trifluoroethyl difluoromethyl ether.

at 5-min intervals into the HSA sample solution. Sequential titrations were performed to ensure full occupancy of the binding sites by loading and titrating with the same ligand without removing the samples from the cell until the titration signal was essentially constant. After the full titration, final concentrations of anesthetics in the HSA cell were approximately 4 mM. The titrations were linked together for data analysis using ConCat32 software distributed from Microcal, Inc. Four separate titrations were performed, including ligand into buffer, buffer into protein, buffer into buffer, and ligand into protein, and the titration corrected accordingly. To ensure data reliability, at least three experiments were performed for each ligand.

Origin 5.0 (Microcal Software, Inc., Northampton, MA) was used to fit thermodynamic parameters to the heat profiles. The following formula was used for data fitting:

$$Q = \frac{nM_t\Delta H V_o}{2} \left[1 + \frac{X_t}{nM_t} + \frac{1}{nKM_t} - \sqrt{\left(1 + \frac{X_t}{nM_t} + \frac{1}{nKM_t} \right)^2 - \frac{4X_t}{nM_t}} \right],$$

where K is the binding constant, n is the number of sites,

V_o is the active volume involved in interaction, M_t is the total concentration of protein in V_o , X_t is the total concentration of ligand, ΔH is the molar heat of ligand binding, and Q is the total heat content of the solution contained in V_o (determined relative to zero for the unliganded species) at fractional saturation. The process of fitting experimental data then involves iterative improvement of initial values of n, K, and ΔH by standard Marquardt methods to minimum chi-square values.

Electrostatic interactions in protein binding sites can be influenced by salt concentration. Increased concentrations of charged ions compete with the ligand for charged residues in the cavity and thus reduce apparent ligand affinity if dependent on that interaction. Therefore, ITC experiments were performed at 130 mM NaCl and at 500 mM NaCl with 20 mM NaHPO₄ and a pH of 7.0.

Enflurane, Isoflurane, and Propofol Competition

To test for overlapping binding sites, competition experiments were performed using ITC in a buffer condition of 130 mM NaCl and 20 mM NaHPO₄, with a pH of 7.0. For competition between isoflurane and enflurane, 0.075 mM HSA in the sample cell was titrated with isoflurane or enflurane followed by enflurane or isoflurane, respectively.

Because the HSA crystallographic binding sites for propofol have been recently reported⁷ and confirmed under solution conditions,⁵ propofol was used as a probe of haloether location by using competition experiments. HSA (0.015 mM) that had been preequilibrated with 0.5 mM propofol was titrated with propofol, isoflurane, or enflurane. For comparison, we repeated the same titrations into HSA without propofol preequilibration.

Molecular Properties and Protein Structure Analysis

The molecular properties of isoflurane and enflurane were calculated using Molecular Analysis Pro (ChemSW, Inc., Fairfield, CA). Dipole moment and partial charges were calculated using the modified partial equalization of orbital electronegativity method.^{8,9} The sterics of protein pockets or cavities and their lining residues in HSA in the absence (1AO6)¹⁰ and presence of propofol (1E7A)⁷ were calculated using CASTp (a Web-based program to determine cavity information[†]).¹¹ Protein coordinates were obtained from the Protein Data Bank (§).

Statistics

The fitted parameters from ITC enthalpograms are presented as mean \pm SD, and the mean values were compared with unpaired *t* tests using InStat v 3.06 (San Diego, CA). A *P* value less than 0.05 was considered significant.

[†] CASTp. Available at: <http://cast.engr.uic.edu/cast/>. Accessed February 19, 2005.

[§] Protein Data Bank. Available at: <http://www.rcsb.org/pdb>. Accessed February 19, 2005.

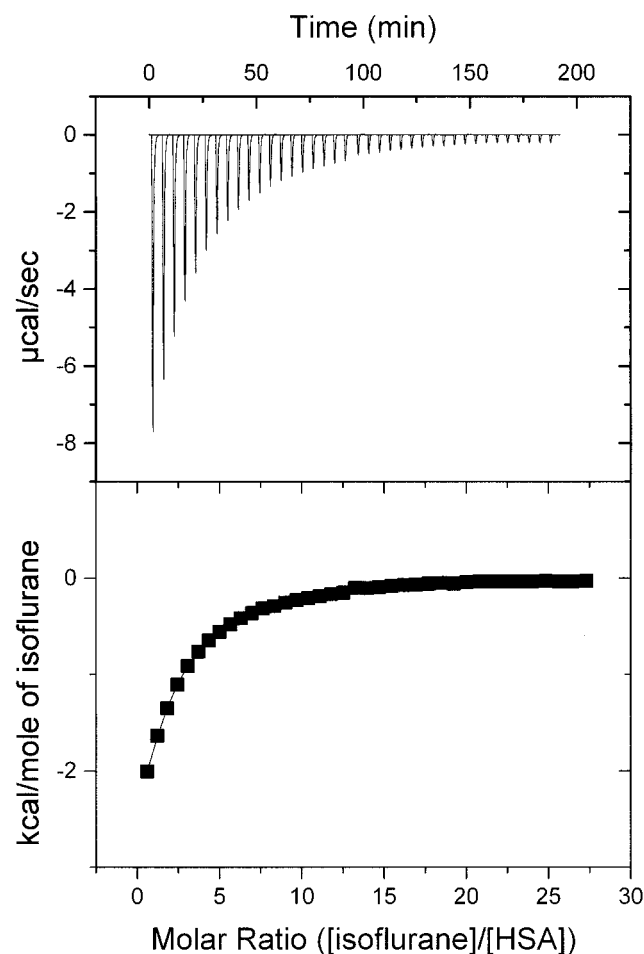


Fig. 2. Isothermal titration calorimetry of isoflurane into human serum albumin (HSA). The *top panel* shows the raw heat data ($\mu\text{cal/s}$) versus time (minutes), after subtraction of the baseline. The *bottom panel* shows normalized integrated data (kcal/mol anesthetic vs. molar ratio). The latter data were used for curve fitting to derive binding and thermodynamic parameters. After a full set of injections, the final isoflurane concentration in the cell was 3.8 mM. Derived parameters are presented in table 1.

Results

Isoflurane and HSA Interaction

The titration of HSA with isoflurane at 130 mM NaCl released heat (fig. 2), and subsequent fitting of the en-

thalpogram with a single-class, variable n binding site model produced a single site with a K_A of $1,400 \text{ M}^{-1}$ ($K_D = 0.7 \text{ mM}$; table 1). At 500 mM NaCl, the affinity for isoflurane decreased significantly ($P < 0.05$; table 1), but mostly through a less favorable entropy term.

Enflurane and HSA Interaction

The enflurane-HSA interaction was also exothermic, with a derived K_A of $1,000 \text{ M}^{-1}$ ($K_D = 1 \text{ mM}$; table 1) for about two identical binding sites. In contrast to isoflurane, 500 mM NaCl did not significantly alter the enflurane-HSA affinity (table 1).

Binding Site Overlap

The heat release diminished to a constant but nonzero level after a full enflurane titration into 0.075 mM HSA. Further titration of this sample with isoflurane was not accompanied by any additional heat release (fig. 3, left). Similarly, heat release diminished to a constant level after full isoflurane titration into 0.075 mM HSA, but in this case, further titration with enflurane was accompanied by further heat release (fig. 3, right).

Preincubation of HSA with 0.5 mM propofol inhibited subsequent heat release with either isoflurane or enflurane titration (fig. 4, left) as compared with HSA without propofol preincubation (fig. 4, right).

Molecular Properties of the Haloethers and the HSA Binding Sites

Isoflurane and enflurane have the same atomic composition, the same molecular weight, and essentially the same molecular volume of approximately 110 \AA^3 , but isoflurane has an almost twofold larger dipole moment than enflurane, 0.7 debye for enflurane versus 2.0 debye for isoflurane.

Isoflurane and enflurane binding was inhibited by propofol, implicating the two propofol binding sites in domain 3 of HSA as haloether binding sites. Both propofol cavities contain charged residues; the one containing tyrosine-411 has charged atoms from four lining residues (NE and NH2 of R410; CE and NZ of K414; CG of R445; and CA, O, CG, and CD of R485; fig. 5), whereas the

Table 1. Thermodynamic Parameters of Interaction of Human Serum Albumin with Isoflurane and Enflurane

| | n | K_A $1 \times 10^3/\text{M}$ | ΔH , kcal/mol | ΔS , $\text{cal}\cdot\text{mol}^{-1}\cdot\text{K}^{-1}$ |
|-------------|-----------------|-----------------------------------|-----------------------|--|
| Enflurane | | | | |
| 130 mM salt | 1.6 ± 0.1 | 1.0 ± 0.02 | -9.3 ± 0.5 | -18.1 |
| 500 mM salt | 1.6 ± 0.2 | 1.1 ± 0.05 | -10.4 ± 1.1 | -21.7 |
| Isoflurane | | | | |
| 130 mM salt | $1.3 \pm 0.1^*$ | $1.4 \pm 0.04^*$ | $-7.8 \pm 0.6^*$ | -11.8 |
| 500 mM salt | 1.3 ± 0.2 | $1.0 \pm 0.05^\dagger$ | -8.6 ± 1.1 | -15.8 |

Data are presented as mean \pm SD. Each sequential titration consists of 38 consecutive automatic injections.

* $P < 0.05$ vs. that of enflurane at 130 mM salt. $^\dagger P < 0.05$ vs. that of isoflurane at 130 mM salt.

ΔH = enthalpy change; ΔS = enthalpy change; K_A = association constant; n = number of binding sites.

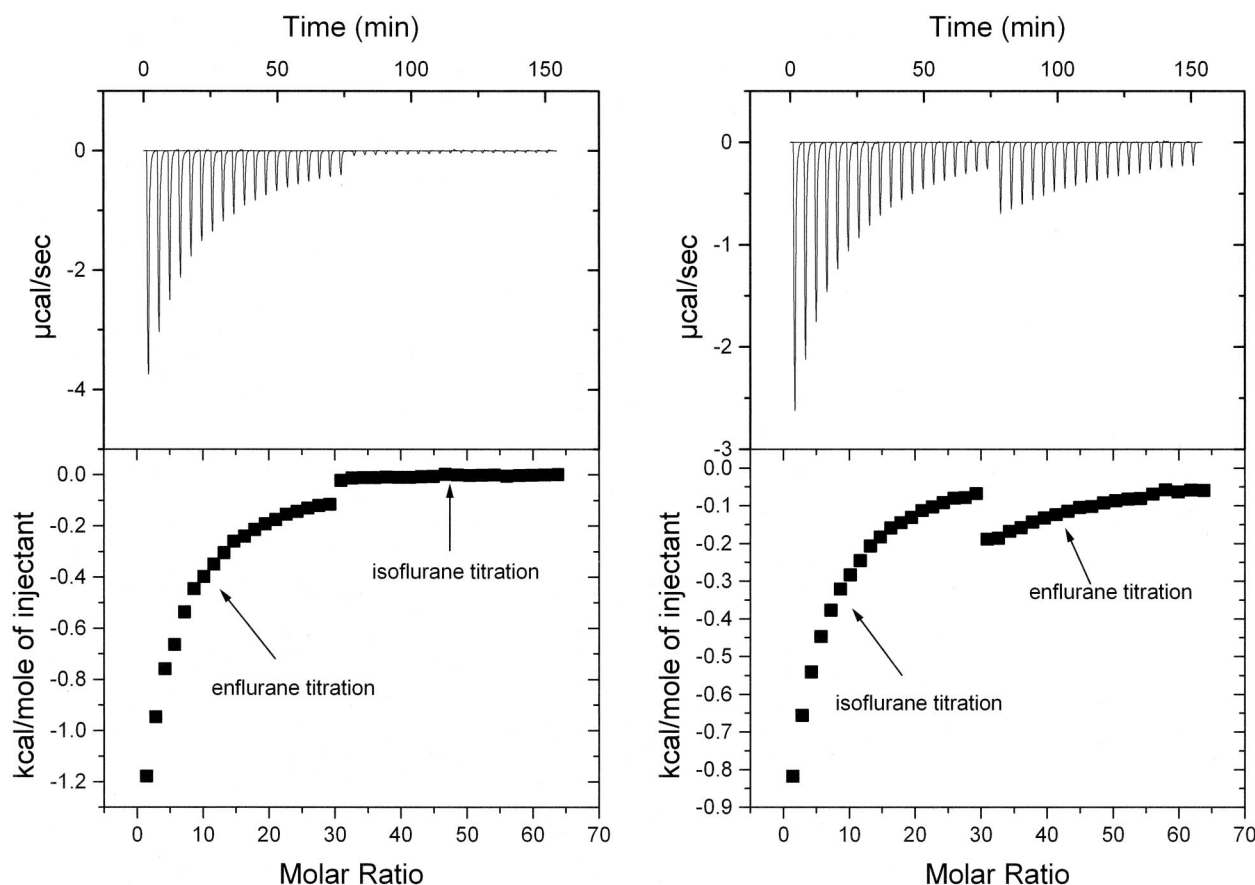


Fig. 3. Isoflurane and enflurane competition. The *left panel* shows heat changes of an initial enflurane titration followed by isoflurane. The lack of additional heat release by isoflurane indicates preoccupation of sites by enflurane. The *right panel* shows the reverse titration, isoflurane followed by enflurane. In this case, the second anesthetic elicited a significant heat response, indicating that some sites for enflurane are not occupied by isoflurane.

other pocket has no charged atoms in the lining (fig. 5). Using CASTp and a 1.4-Å radius probe, we determined the cavity volume in the presence and absence of propofol and found that the site containing tyrosine-411 increased from 340 Å³ in 1A06 to 510 Å³ in 1E7A on propofol binding. The second propofol binding pocket initially consisted of small pockets of only 20–200 Å³ (fig. 5) in the unliganded 1A06 but coalesced to a larger cavity of 810 Å³ in the propofol-bound state (1E7A).

Discussion

The principal finding of this study is that structural isomers of the haloether anesthetics retain sufficient electrostatic identity to select different protein binding sites. Therefore, it is entirely feasible that the differences in the various actions of isoflurane *versus* enflurane are due to occupancy of different binding sites, perhaps on different molecular targets.

Location and Number of Sites

In contrast to the large number of haloalkane binding sites on HSA demonstrated with many approaches,^{5,7,12}

including ITC,⁵ our current ITC experiments indicate that isoflurane has only a single energetically significant binding site. Using mutagenesis, we have previously demonstrated that isoflurane binds at the Y411 binding site of HSA,¹² indicating that this site is the dominant isoflurane binding site. Competition between isoflurane and enflurane indicated that enflurane also binds to the Y411 site. Further confirmation is provided by the propofol competition experiments, because the Y411 site is also a known propofol binding site.^{5,7} Enflurane seems to have an additional site that excludes isoflurane in the concentration range achieved here (up to 4 mM). The observation that propofol binding fully inhibits haloether binding suggests that the additional enflurane binding site is the second site for propofol, also in domain three.

Binding Site Character

It is predicted that the relation between cavity volume and ligand molecular volume plays a role in binding site selectivity. Using the short dimension of these molecules of approximately 5.8 Å and using CASTp with this probe size (instead of the normal 1.4 Å), six cavities in HSA

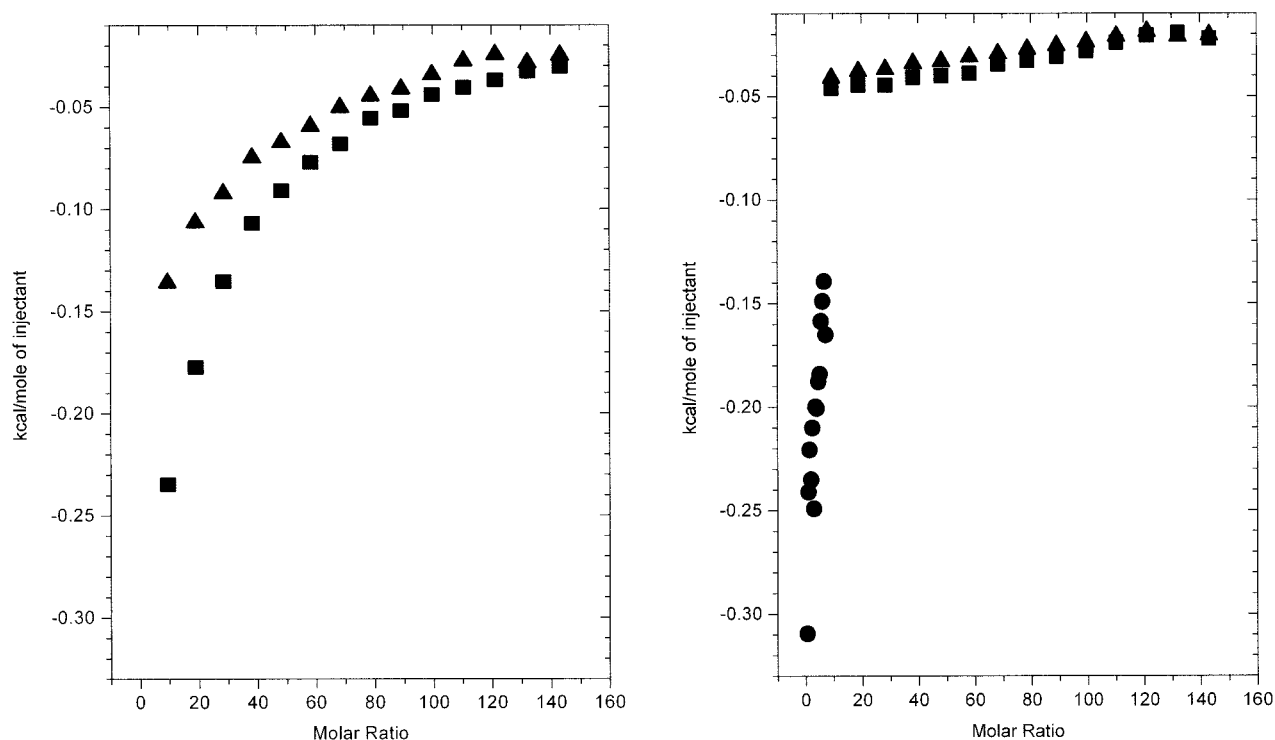


Fig. 4. Propofol and haloether competition. The *left panel* shows enflurane and isoflurane titration into 0.015 mM human serum albumin without previous equilibration with propofol. The *right panel* shows propofol, enflurane, and isoflurane titration into 0.015 mM human serum albumin that had been preequilibrated with 0.5 mM propofol. The heat release due to enflurane and isoflurane seen in the *left panel* was fully inhibited by propofol preequilibration, indicating that the binding sites of both enflurane and isoflurane were preoccupied by propofol.

(1E7B) are large enough for isoflurane or enflurane. Therefore, binding site selectivity must rely on features other than volume. Although the same atoms comprise the two haloether molecules, the different arrangement produces a different shape (fig. 1), and so the corresponding shape of the cavity might also contribute; few are expected to match that of the anesthetic perfectly. However, this may be less important than supposed because of the dynamic nature of proteins and their cavities—in part reflected by the substantial change in

cavity volume noted in the analysis of x-ray diffraction data. A clear difference between enflurane and isoflurane is the permanent dipole moment; therefore, dipole-dipole interactions might contribute to selectivity. Consistent with this possibility, there are four positively charged residues lining the isoflurane binding site with the charged side chain atoms forming the pocket surface (fig. 5). The enhanced enthalpy per site for isoflurane as compared with enflurane also supports this idea. Even further support comes from the observation that in-

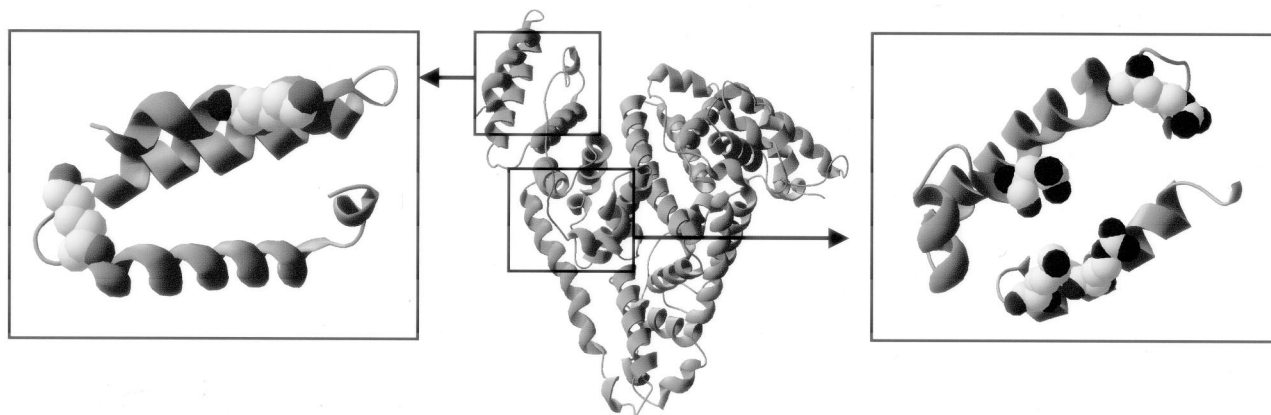


Fig. 5. Location and different character of the anesthetic binding sites in domain 3 of human serum albumin. Only charged lining residues are shown. On the right, note the four charged residues in the common site for halothane, enflurane, isoflurane, and propofol. The other site for propofol and enflurane has only two charged residues, and the polar atoms do not line the cavity. In *both panels*, the cavities lie in an otherwise similar interhelical space.

creased salt concentration reduces isoflurane affinity, presumably *via* charge “screening.” Although two positively charged residues line the additional enflurane binding site, the charged atoms are not part of the pocket surface, suggesting that this cavity is not as polar as the other. Propofol, being intermediate in dipole moment (1.6 debye), binds both cavities, although consistent with the above, the crystal data suggests higher occupancy of the Y411 cavity.

Binding Energetics

Using ^{19}F nuclear magnetic resonance spectroscopy^{13,14} and competitive photoaffinity labeling,¹⁵ isoflurane has been shown to bind to bovine serum albumin with K_D values of 1.36–1.5 mM. Using a very different method, ITC, we found comparable overall affinity for isoflurane (0.7 mM). It is important to note that although ITC can determine the full thermodynamic profile for bimolecular interactions, it is difficult to unambiguously derive all parameters when the Wiseman c parameter¹⁶ (the product of the protein concentration and K_A) is less than approximately 10. Nevertheless, recent studies have indicated reliable parameter estimation using ITC in low-affinity systems.¹⁷ ITC has the distinct advantage of being unbiased with respect to any particular “reporter” feature of either ligand or target—heat change is a feature of all energetically significant interactions. Thus, heat is released (exothermic; negative ΔH) or absorbed (endothermic; positive ΔH) in direct proportion to the extent and strength of interaction that occurs. For example, the interactions of inhaled anesthetics with a designed anesthetic-binding, four-helix bundle protein were exothermic.⁶ Further, the anesthetic–firefly luciferase interaction is exothermic,¹⁸ as is the chloroform–bovine serum albumin interaction.¹⁹ Finally, we have shown that both halothane and propofol binding to HSA is exothermic.⁵ However, the Overton-Meyer relation (positive correlation between hydrophobicity and potency) and what limited structural information exists indicate that the dominant interactions are hydrophobic in nature. Hydrophobic interactions are generally accompanied by a very small ΔH —most of the binding energetics being driven by favorable changes in entropy. However, the consistently negative ΔH in this and past studies suggests that some polar interactions must contribute to anesthetic binding. It is perhaps surprising that the HSA interaction with both haloethers has a considerably negative ΔH value, suggesting that even with the smaller dipole moment, other weak electrostatics, like van der Waals interactions, must play a role in enflurane binding. Nevertheless, in the case presented here, it is highly probable that the permanent dipole of the anesthetic molecule provides the force responsible for site selectivity.

The observed selectivity may have pharmacologic significance. The ability of several general anesthetics (halo-

thane, isoflurane, enflurane, and propofol) to bind at the same site in domain 3 of HSA suggests the possibility that this site bears resemblance to sites in the central nervous system that contribute to the effect that all of these drugs have in common: anesthesia. The additional site for enflurane might resemble those responsible for its different effects.^{20–24} For example, enflurane is associated with epileptiform activity in some patients,²⁵ and other inhaled drugs with this property have a tendency to be even more apolar. It is tempting to speculate that the lower potency of enflurane as compared with the less hydrophobic isoflurane, a violation of the Overton-Meyer relation, is in part due to occupancy of functionally opposed sites—in either the same or different targets.

In summary, two binding sites of different character exist in HSA for the haloether anesthetics. One site is more polar and prefers isoflurane, presumably because of its larger dipole. The second site is less polar and binds only enflurane. Therefore, in addition to molecular volume and hydrophobic surface area, weak polar interactions confer considerable binding selectivity, which may underlie differences in drug action.

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