^{19}F Nuclear Magnetic Resonance Investigation of Stereoselective Binding of Isoflurane to Bovine Serum Albumin

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ABSTRACT Whether proteins or lipids are the primary target sites for general anesthetic action has engendered considerable debate. Recent in vivo studies have shown that the S(+) and R(−) enantiomers of isoflurane are not equipotent, implying involvement of proteins. Bovine serum albumin (BSA), a soluble protein devoid of lipid, contains specific binding sites for isoflurane and other anesthetics. We therefore conducted ^{19}F nuclear magnetic resonance measurements to determine whether binding of isoflurane to BSA was stereoselective. Isoflurane chemical shifts were measured as a function of BSA concentration to determine the chemical shift differences between the free and bound isoflurane. K_D was determined by measuring the ^{19}F transverse relaxation times (T_2) as a function of isoflurane concentration. The binding duration was determined by assessing increases in $1/T_2$ as a result of isoflurane exchanging between the free and bound states. The S(+) and R(−) enantiomers exhibited no stereoselectivity in chemical shifts and K_D values (K_D = 1.3 ± 0.2 mM, mean ± SE, for S(+), R(−), and the racemic mixture). Nonetheless, stereoselectivity was observed in dynamic binding parameters; the S(+) enantiomer bound with slower association and dissociation rates than the R(−).

INTRODUCTION

One of the prevailing theories on molecular mechanisms of general anesthesia hypothesizes that proteins, rather than lipids, are the primary target sites for anesthetic action (Franks and Lieb, 1994). Principal findings in support of this theory include (1) that the photon emission from firefly luciferase, a lipid-free protein, can be inhibited by a variety of general anesthetics at their respective clinical concentrations (Franks and Lieb, 1984); (2) that some important ligand-gated ion channels, e.g., nicotinic acetylcholine (ACh) receptors (Firestone et al., 1986, 1994; Dilger et al., 1992; Dilger and Liu, 1992) and γ-amino butyric acid ($GABA_A$) receptors (Jones et al., 1992; Lin et al., 1992, 1993b; Nakahiro et al., 1989; Wakamori et al., 1991), are sensitive to volatile anesthetics at clinical concentrations; (3) that bovine serum albumin (BSA), another lipid-free soluble protein, contains a limited number of saturable binding sites for volatile anesthetics such as halothane and isoflurane (Dubois and Evers, 1992; Dubois et al., 1993); and (4) that optically pure enantiomers of isoflurane exhibit different anesthetic potencies in vivo (Harris et al., 1992; Lysko et al., 1994). Although no protein in the central nervous system (CNS) has yet been positively identified as the target site for general anesthesia, numerous examples exist wherein the activity of a protein is profoundly affected by a single small effector molecule. It is thus conceivable that the function of some crucial proteins in the CNS can be altered by anesthetic binding. This concept has motivated extensive investigation to determine anesthetic-protein interactions at the cellular and molecular levels (Abadji et al., 1993; Dickinson et al., 1993; Dilger and Brett, 1991; Dilger and Liu, 1992; Dilger et al., 1993, 1994; Dubois and Evers, 1992; Hall et al., 1994; Harrison et al., 1993; Jones et al., 1992; Jones and Harrison, 1993; Lin et al., 1993a,b; Zimmerman et al., 1994), despite the confounding possibility that anesthetic actions on proteins may result from indirect actions mediated by changes in the surrounding lipids (Fraser et al., 1990; Abadji et al., 1993).

Stereoselective anesthetic action is one of the few tests for the protein theory; stereoselectivity usually suggests direct and specific receptor-mediated action (Franks and Lieb, 1991, 1994; Hall et al., 1994; Harris et al., 1992, 1994; Lysko et al., 1994; Moody et al., 1993, 1994; Eckenhoff and Shuman, 1993). Therefore, it is important to evaluate the intrinsic ability of anesthetic enantiomers to interact with pure proteins. In this study, we examined the binding of the two optical enantiomers of isoflurane to BSA, using ^{19}F nuclear magnetic resonance (NMR) spectroscopy and gas chromatography (GC). We predicted that the binding would be stereoselective, as suggested by other physical (Eckenhoff and Shuman, 1993) and indirect (Franks and Lieb, 1991; Jones and Harrison, 1993) methods.

MATERIALS AND METHODS

Materials

Racemic isoflurane was purchased from Abbott Laboratories (North Chicago, IL). Optically pure enantiomers, S(+) and R(−), were generously provided by J. Vernice of Anaquest (Murray Hill, NJ). Lyophilized BSA (fraction V, fatty acid free) was obtained from Sigma Chemical Co. (St. Louis, MO). Other chemicals, of analytical reagent grade, were purchased from Sigma and Mallinckrodt (Paris, KY). All agents were used without further purification.

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Preparation of saturated isoflurane solution

Saturated solutions of S(+), R(−), or racemic isoflurane were prepared by mixing, at 4°C and in gas-tight vials, an excess amount (typically 130 μl) of neat isoflurane in 50 ml of Hepes buffer solution, containing 135 mM KCl, 15 mM sodium gluconate, 1 mM EDTA, 0.75 mM $CaCl_2$, and 10 mM glucose. Saturation was confirmed by the presence of residual neat isoflurane and by repeated GC measurements showing constant isoflurane concentrations of 14.2 ± 0.1, 14.1 ± 0.1, and 14.4 ± 0.2 mM (mean ± SD) for S(+), R(−), and the racemic mixture, respectively, at room temperature.

Preparation of BSA solutions

Lyophilized BSA (M_r = 66.2 × 10^3) was dissolved in the Hepes buffer by gentle magnetic stirring at 4°C. The concentrations used in NMR and GC measurements ranged from 0.75 mg/ml (11.3 μM) to 90 mg/ml (1.4 mM). To separate specific binding at the fatty-acid-replaceable sites from nonspecific binding, NMR measurements (see below) were also carried out to determine any residual isoflurane binding to BSA when the latter was either acidified with HCl to pH 2.5 or complexed to oleic acid at an oleic-acid-to-BSA molar ratio of 6 to 1. To prepare the oleic-acid-BSA complex, a measured amount of sodium oleate was first dissolved in distilled water and then added to a given BSA solution, as described by Spector (1986).

GC techniques and X_b calculations

Saturated isoflurane solution, BSA stock solution, and Hepes buffer were mixed in gas-tight vials such that the final aqueous volumes occupied one-half of the vial's total volume (1 ml). The mixtures were allowed to equilibrate at room temperature for 24 h. The BSA concentrations ranged from 0 to 75 mg/ml. The nominal initial isoflurane concentration was 4 mM, which, after equilibration, resulted in a final aqueous isoflurane concentration comparable to that used in the corresponding NMR studies (see below). Aliquots (2 μl) of liquid sample were withdrawn using a gas-tight syringe (Hamilton) and were quantified by GC (Perkin-Elmer 8500; Poropak P resin; 150°C).

Based on the GC measurements, the fraction of isoflurane bound to BSA, X_b, was calculated using the method described in detail by Dubois and Evers (1992).

NMR techniques

All samples for NMR were prepared by mixing measured amounts of saturated isoflurane solution, BSA stock solution, and the Hepes buffer directly inside 5-mm, high precision NMR tubes (Wilmad Glass Co., Buena, NJ), which were capped and almost completely filled to minimize volatile anesthetic loss from the aqueous phase. A tiny air bubble, <1% of the tube volume, was intentionally left in the tubes for the purpose of inversion mixing. All ^{19}F NMR experiments were conducted using an Otsuka-Chemagnetics (Fort Collins, CO) CMXW-400 spectrometer operating at 377.168 MHz. The resonance frequencies of isoflurane were measured as chemical shifts (in units of parts per million, ppm) relative to an external reference of diluted CF_3COOH solution contained in a 10-mm NMR tube coaxial to the 5-mm sample tubes. The transverse relaxation time (T_2), which characterizes the motional restriction of isoflurane molecules, was measured using the conventional Carr-Purcell-Meiboom-Gill (CPMG) spin-echo method (Meiboom and Gill, 1958). In this method, a 90° radiofrequency pulse is given to prepare the ^{19}F nuclei, which are then refocused repeatedly by a series of 180° pulses to create a train of spin echoes. The echo maxima were digitized and T_2 calculated from nonlinear regression of the echo maxima using exponential decay functions. In our experiments, the pulse lengths for the 90° and 180° pulses were 27 μs and 54 μs, respectively. The time between the consecutive 180° pulses, τ_{cp}, ranged from 60 μs to 4 ms.

Binding analysis by NMR measurements

There are at least two possible environments for isoflurane in a BSA solution: a bound environment and an aqueous, free environment. When isoflurane exchanges fast enough between these environments, resonance peaks that characterize each of the environments coalesce to give rise to a single resonance (Trudell and Hubbell, 1976). The observed chemical shifts and T_2 of this coalesced resonance contain weighted contributions from the two environments and thus can be used for binding analysis (Fraenkel et al., 1990; Dubois and Evers, 1992).

Chemical shifts are sensitive indicators of the chemical environment in which resonating nuclei reside. The limiting shift for the isoflurane in the bound environment is thus characteristic of the binding sites. Let δ_f and δ_b be the limiting shifts of the free and bound isoflurane, respectively, and δ be the observed shift. It can be shown that

$$\frac{1}{\delta - \delta_f} = \frac{1}{\delta_b - \delta_f}\left(1 + \frac{K_D}{[R]}\right) \tag{1}$$

where [R] is the equilibrium BSA concentration, and K_D is the dissociation constant of the isoflurane-BSA complex. Thus, if chemical shifts are measured as a function of BSA concentration at a fixed isoflurane concentration, the weighting for the two-site averaging is shifted toward the bound environment. At high BSA concentrations, [R] can be approximated by the initial BSA concentration, $[R]_0$. By extrapolating the reciprocal of the measured relative chemical shifts to $1/[R]_0 \rightarrow 0$ (corresponding to $X_b = 1$), the limiting chemical shift for the bound isoflurane can be determined.

Theories have been developed (Dwek, 1973) to account for T_2 relaxation in a two-site system where relaxation due to exchange between the sites is not negligible. It has been shown (Allerhand and Gutowsky, 1965) that when the CPMG method is used, the observed T_2 is a function of the 180°-180° interpulse delay τ_{cp}:

$$\frac{1}{T_2} = \frac{1 - X_b}{T_{2f}} + \frac{X_b}{T_{2b} + \tau_b} + \tau_b X_b (1 - X_b)(\delta\omega)^2 f(\tau_{cp}) \tag{2}$$

where T_{2f} and T_{2b} are the intrinsic T_2 of isoflurane in the free and the bound states, respectively, $\delta\omega = 2\pi(\delta_b - \delta_f)$ is the chemical shift difference between the free and bound isoflurane, τ_b is the lifetime of isoflurane in the bound state, and $f(\tau_{cp})$ is a hyperbolic tangent:

$$f(\tau_{cp}) = 1 - \frac{2\tau_b}{\tau_{cp}} \tanh \frac{\tau_{cp}}{2\tau_b} \tag{3}$$

After X_b and $\delta\omega$ are determined, nonlinear regression of measured $1/T_2$ as a function of $1/\tau_{cp}$ using Eq. 2 will generate the best estimates of T_{2b} and τ_b.

Initial values of the fitting parameters often influence the goodness of nonlinear regression (Glantz and Slinker, 1990a). For Eq. 2, two limiting cases can be considered for the initial estimate of τ_b. In the slow pulsing limit, $\tau_{cp} \gg \tau_b$, $f(\tau_{cp}) \rightarrow 1$, and Eq. 2 becomes

$$\frac{1}{T_2} = \frac{1 - X_b}{T_{2f}} + \frac{X_b}{T_{2b} + \tau_b} + \tau_b X_b (1 - X_b)(\delta\omega)^2 \tag{4}$$

In the fast pulsing limit, $\tau_{cp} \ll \tau_b$, $f(\tau_{cp}) \rightarrow 0$, and

$$\frac{1}{T_2} = \frac{1 - X_b}{T_{2f}} + \frac{X_b}{T_{2b} + \tau_b} \tag{5}$$

Equations 4 and 5 define the upper and lower limits of $1/T_2$. The difference between the two limits is proportional to τ_b.

In this study, T_{2b} and τ_b were determined at an isoflurane concentration of 1.65 mM and BSA concentrations of 0.75, 1.75, and 6.00 mg/ml. Repeated T_2 measurements were made with variable τ_{cp} ranging from 60 μs to 4 ms. With the knowledge of X_b from the GC measurements, $\delta\omega$ from

the chemical shift measurements, and T_{2f} from the T_2 measurements in the BSA-free buffer, the best estimates of T_{2b} and τ_b were obtained from nonlinear regression using Eq. 2.

In addition to T_{2b} and τ_b, Eq. 5 can be rearranged to determine K_D. By expressing X_b in terms of K_D, it follows that (Dubois and Evers 1992)

$$T_{2p} = \frac{T_{2b} + \tau_b}{[R]_0}[A]_0 + \frac{T_{2b} + \tau_b}{[R]_0} K_D \qquad (6)$$

where $[A]_0$ is the initial concentration of isoflurane and

$$T_{2p} = (1/T_2 - 1/T_{2f})^{-1} \qquad (7)$$

is the additional relaxation due to binding. Hence, $T_{2p}[R]_0$ is a linear function of $[A]_0$, having a slope of $T_{2b} + \tau_b$ and an intercept on the x axis of $-K_D$.

In this study, K_D was determined by repeated T_2 measurements at six different isoflurane concentrations (ranging from 0.82 to 4.94 mM) and three BSA concentrations (0.75, 1.75, and 6.00 mg/ml). The echo time τ_{cp} was fixed at 100 μs for these experiments. A total of 78 samples were measured: 24 for S(+), 31 for R(−), and 23 for the racemic mixture. Depending on the signal-to-noise ratio of each measurement, the number of acquisitions averaged ranged from 32 to 256. For most of the samples, the measurements were repeated two or three times.

To determine the K_D at the fatty-acid-replaceable sites (i.e., K_D of specific binding), the T_{2f} values used in Eq. 7 for T_{2p} calculation were those obtained separately at the above three BSA concentrations when the BSA was complexed with oleic acid (oleic acid:BSA = 6:1).

Statistical analysis

Student's t-test was used to compare X_b of the two enantiomers for a significance level of $p = 0.05$. To compare K_D and $T_{2b} + \tau_b$ between S(+) and R(−), the t statistics were calculated for the x intercepts and the slopes of the two regression lines using Eq. 6. The t statistic is the ratio of the difference of the two slopes or intercepts to the standard error of the difference. Because different numbers of samples were measured for the S(+) and R(−), the standard error of the difference is computed by using a pooled variance, which is a weighted average based on the degrees of freedom in each case (Glantz and Slinker, 1990b; Glantz, 1992). The calculated t was then compared with the critical value of t, t_c, for the corresponding degrees of freedom in the measurements. A p value of less than 0.05 was considered as statistically significant.

RESULTS

Chemical shift measurements at various BSA concentrations revealed no differences between the S(+) and R(−) enantiomers or the racemic mixture. Fig. 1 *A* shows representative ^{19}F-NMR spectra of 1.65 mM S(+), R(−), and racemic isoflurane (peaks at −4.24 and −10.11 ppm) in 75 mg/ml BSA solutions. All shifts were measured relative to the common external reference (CF_3COOH, peaks at 0 ppm) in the 10-mm coaxial tube to determine the chemical shift differences. The inset to Fig. 1 *a* expands the region of the trifluoromethyl resonance, showing essentially the same chemical shifts for the two enantiomers and the racemic mixture. This was also true at other BSA concentrations studied, ranging from 0 to 90 mg/ml (corresponding to X_b of 0 to 0.7; Dubois and Evers, 1992).

Fig. 1 *b* depicts the reciprocal of relative changes in chemical shift as a function of the reciprocal of the initial BSA concentration for 1.65 mM S(+), R(−), and racemic isoflurane. The solid lines, which overlap one another, are linear least-squares fit to the data (Eq. 1). The y intercepts yield $\delta\omega/2\pi = \delta_b - \delta_f = 0.50 \pm 0.02$ ppm. Although the

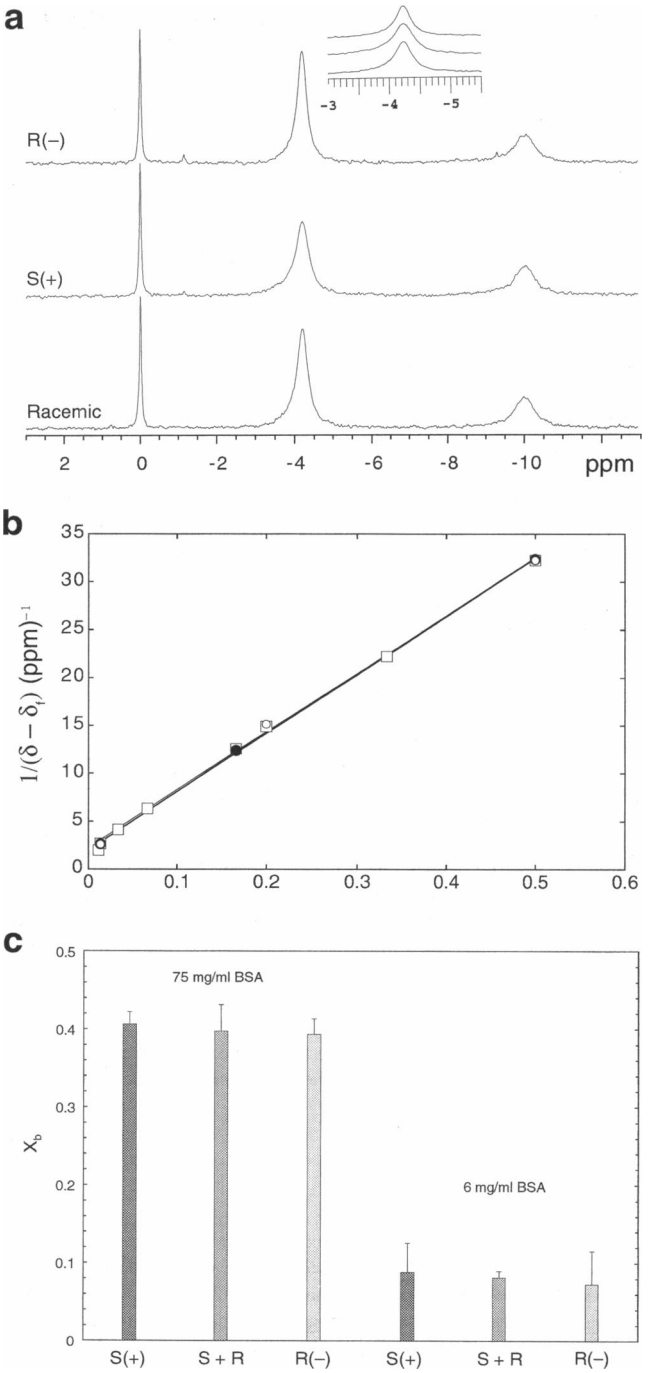

FIGURE 1 (*a*) Representative ^{19}F NMR spectra of 1.65 mM S(+), R(−), and racemic isoflurane in 75-mg/ml BSA solutions. The inset expands the region of isoflurane's trifluoromethyl resonance near −4.24 ppm, showing the same chemical shifts relative to the common external reference (CF_3COOH, peaks at 0 ppm). (*b*) Plot of $1/(\delta - \delta_f)$ as a function of $1/[R]_0$. Extrapolation of $1/[R]_0$ to 0 yields $\delta_b - \delta_f = 0.50 \pm 0.02$ ppm (Eq. 1). ●, S(+); ○, R(−); □, racemic. (*c*) Comparison of fraction of bound isoflurane, X_b, for 1.65 mM S(+), R(−), and racemic isoflurane in 6- and 75-mg/ml BSA solutions, showing no significant difference in X_b between S(+) and R(−). Error bars indicate standard deviations ($n = 7$).

slopes are proportional to K_D, difficulties in determining equilibrium BSA concentrations exclude the use of this method for accurate K_D measurement. Nevertheless, the same slopes for the S(+), R(−), and racemic isoflurane suggest that the K_D is the same for the two enantiomers.

The fraction of bound isoflurane (X_b) was determined at various BSA concentrations. Fig. 1 *c* compares the X_b for 1.65 mM of either S(+), R(−), or racemic isoflurane in both 6- and 75-mg/ml BSA solutions. There was no significant difference in X_b between the two enantiomers ($p > 0.05$, $n = 7$).

Fig. 2 shows K_D analysis using Eq. 6 from repeated T_2 measurements at different initial isoflurane concentrations. To determine K_D at only the fatty-acid-replaceable binding sites, T_{2p} was calculated by replacing the T_{2f} in Eq. 7 with the T_2 measured in oleic-acid-complexed BSA solutions (oleic acid:BSA = 6:1), so that any additional relaxation caused by nonspecific (i.e., oleic-acid-insensitive) binding was canceled. The inset to Fig. 2 depicts individual measurements at BSA concentrations of 0.75, 1.75, and 6 mg/ml for S(+), R(−), and racemic isoflurane, whereas the main plot summarizes all $T_{2p}[R]_0$ as a function of initial isoflurane concentrations, where $[R]_0$ is the nominal initial BSA concentrations in mM. The solid lines in the figure are linear regression to the data. According to Eq. 6, the slope and the intercept on the *x* axis of the regression lines are, respectively, the best estimates of $T_{2b} + \tau_b$ and $-K_D$. These parameters are summarized in Table 1. Student's *t*-test on the two regression lines for the S(+) and R(−) enantiomers showed significant difference in the slopes ($t_{slope} = 17.56$, $p < 0.001$) but no significant difference in the intercepts ($t_{intercept} = 0.33$, $p > 0.5$).

TABLE 1 Comparison of S(+), R(−), and racemic isoflurane binding to BSA

	$(T_{2b} + \tau_b)_{specific}$ (ms),* Eq. 6	$(K_D)_{specific}$ (mM),* Eq. 6	T_{2b} (ms),‡ Eq. 2	τ_b (μs),‡ Eq. 2
S(+)	1.18 ± 0.05	1.29 ± 0.20	3.7 ± 0.1	230 ± 17
S + R	1.27 ± 0.08	1.26 ± 0.26	4.4 ± 0.1	215 ± 10
R(−)	1.37 ± 0.06	1.28 ± 0.21	5.2 ± 0.3	191 ± 13

*Data shown are best estimates ± standard error of the estimates from linear regression, Eq. 6. The subscript specific is used to indicate that the measured parameters are for binding at the fatty-acid-replaceable sites.
‡Data shown are mean ± SD of four separate measurements at BSA concentrations of 0.75 ($n = 1$), 1.75 ($n = 1$), and 6 ($n = 2$) mg/ml.

Fig. 3 depicts measured $1/T_2$ as a function of interpulsing rate $1/\tau_{cp}$ for 1.65 mM isoflurane at three BSA concentrations (0.75, 1.75, and 6 mg/ml). The solid lines are nonlinear regressions of the data using Eq. 2. In the regression, the limiting chemical shift of 190 Hz, determined from extrapolation of measured chemical shifts at different BSA concentrations, and T_{2f} measured in BSA-free buffer solutions (1.51 ± 0.02, 1.56 ± 0.03, and 1.52 ± 0.02 s for S(+), R(−), and racemic isoflurane, respectively), were used. Because no significant difference in X_b was found between S(+) and R(−) enantiomers, X_b values measured with the racemic isoflurane at the three BSA concentrations (see above, also Dubois and Evers, 1992) were used in the regression. The results of the fitting are also given in Table 1. Although the apparent equilibrium dissociation constants were the same within experimental errors for the two enantiomers, the binding dynamics, as characterized by T_{2b} and τ_b, was significantly different ($p < 0.01$).

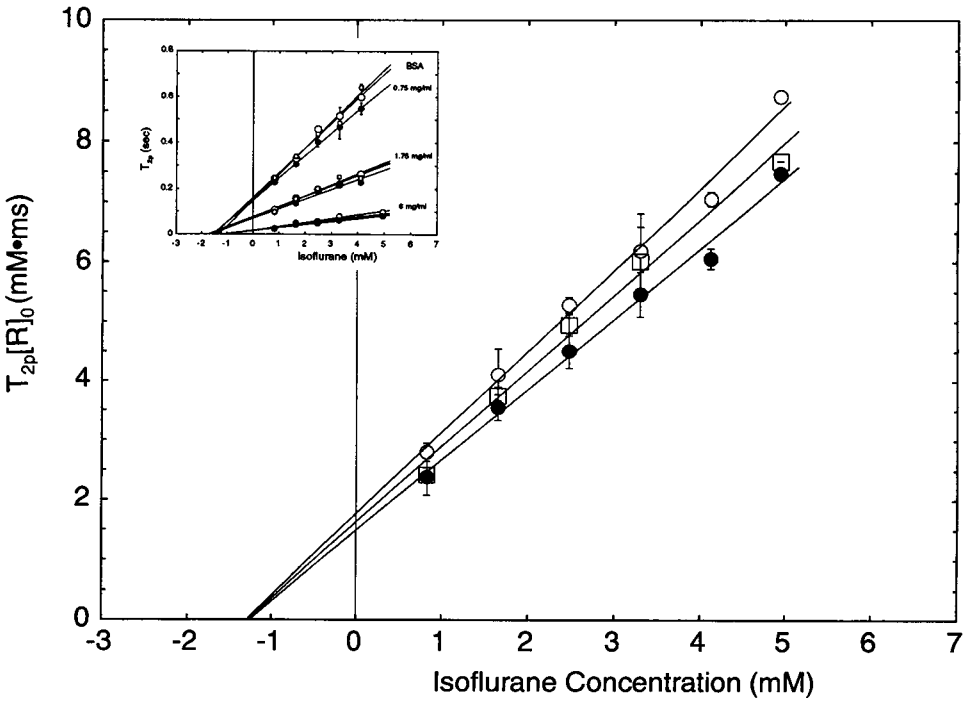

FIGURE 2 K_D analysis based on repeated T_2 measurements (Eq. 6). *Inset*: T_{2p} is plotted as a function of initial isoflurane concentration in 0.75-, 1.75-, and 6.00-mg/ml BSA solutions. *Main plot*: Summary of $T_{2p}[R]_0$ as a function of initial isoflurane concentration. The T_{2f} values used for T_{2p} calculation were the T_2 values measured in the oleic-acid-complexed BSA solutions of the respective concentrations. Error bars are standard deviations (SD) of the measurements at each isoflurane concentration. Where there are no error bars, the SD is smaller than the size of the symbol. Solid lines are linear regression to the data. The slope and the intercept on the *x* axis of the regression lines are, respectively, the best estimates of $T_{2b} + \tau_b$ and $-K_D$ (Eq. 6). ●, S(+); ○, R(−); □, racemic.

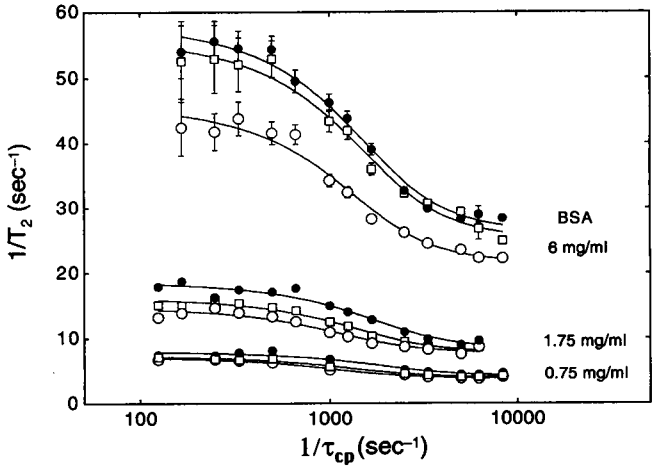

FIGURE 3 Dependence of $1/T_2$ of trifluoromethyl resonance of S(+) (●), R(−) (○), and racemic (□) isoflurane (1.65 mM) on pulse repetition rate, $1/\tau_{cp}$. BSA concentrations are as indicated. (n = 1 for 0.75 and 1.75 mg/ml; n = 2 for 6 mg/ml. Error bars indicate SD.) At a given X_b, the $\Delta(1/T_2)$ between the fast-pulsing ($1/\tau_{cp} \rightarrow \infty$) and the slow-pulsing ($1/\tau_{cp} \rightarrow 0$) limits is proportional to τ_b. Note the difference in limiting $\Delta(1/T_2)$ between S(+) and R(−) at the same BSA concentrations. Solid lines are nonlinear regression of data using Eq. 2, yielding the best estimates of τ_b and T_{2b}.

DISCUSSION

Because there were no chemical shift differences between the S(+) and R(−) enantiomers over a wide range of BSA concentrations, extrapolation of $1/[R]_0 \rightarrow 0$ ($X_b \rightarrow 1$) resulted in the same limiting shifts for the two enantiomers in the bound state. This finding strongly suggests that the S(+) and R(−) enantiomers bind to the same sites (i.e., the same microenvironments) on BSA. Moreover, because the K_D values were the same, the equilibrium concentrations of the two enantiomers at these sites must also have been the same. Therefore, for these parameters, our results showed no stereoselectivity in isoflurane binding to BSA.

Our experiments, however, did reveal stereoselectivity in dynamic parameters; the S(+) enantiomer had a faster relaxation rate (smaller T_{2b}) and a longer lifetime (greater τ_b) in the bound state than the R(−). Under our experimental conditions, T_2 reflected motional restriction of the molecules bearing the nuclei under investigation (e.g., ^{19}F). In general, a shorter T_2 indicates a less mobile environment for the nuclei. Because T_{2b} for S(+) was significantly shorter than for R(−), motion while bound was more restricted for S(+) than for R(−). This was true for specific binding alone, as assessed from the slopes in Fig. 2, or for the combination of specific and nonspecific binding, as calculated from the nonlinear regression in Fig. 3. It should be pointed out that for both enantiomers, motion was more restricted at the specific binding sites than at the nonspecific sites, as T_{2b} obtained from Fig. 2 was shorter than that from Fig. 3 (Table 1).

Examination of the lifetime of the S(+) and R(−) enantiomers at the low affinity (nonspecific) binding sites (at pH 2.5) showed no significant difference in τ_b. If a τ_b value of 60 μs is used for low affinity binding (Dubois and Evers, 1992) and 30% of the total binding is assumed to occur at the low affinity sites (Dubois and Evers, 1992), then the τ_b values for high affinity binding can be calculated, using the values listed in Table 1, to be $((231 - 60 \times 0.3)/0.7) = 304$ μs and $((191 - 60 \times 0.3)/0.7) = 247$ μs for the S(+) and R(−) isoflurane, respectively. Thus, at the high affinity sites, the dissociation rate constant, k_{off}, can be estimated to be 3.3×10^3 and 4.1×10^3 s^{-1}, and the association rate constant, $k_{on} = k_{off}/K_D$, to be 2.6×10^6 and 3.2×10^6 M^{-1} s^{-1} for the S(+) and R(−) isoflurane, respectively. Because k_{off} measures the strength of the binding, these results are consistent with the shorter T_{2b} (less mobility) found for the S(+) enantiomer.

K_D measurement based on Eq. 6 requires $\tau_{cp} \ll \tau_b$. In our experiment, a τ_{cp} of 100 μs was used, which was not significantly smaller than the τ_b for the specific binding estimated above. Possible errors introduced by using Eq. 6 can be estimated using Eqs. 2 and 3. For τ_{cp} = 100 μs and τ_b = 250 μs, it can be calculated from Eq. 3 that $f(\tau_{cp}) \approx 0.01$, and the relative error in $1/T_{2p}$ by neglecting the third term in Eq. 2 is <0.4%.

Using photoaffinity labeling, Eckenhoff and Shuman (1993) found that S(+) isoflurane was 50% more effective than R(−) isoflurane in inhibiting halothane binding to BSA. They also found that inhibition by the racemic mixture of isoflurane was very similar to that by S(+). Because the inhibition constant K_I is a measure of K_D for the dissociation of the isoflurane-BSA complex, their results might appear to contradict ours. However, photoaffinity labeling is an intrinsically nonspecific method for any binding with K_D of ~1 mM or greater (Ruoho et al., 1984). In fact, the ratio of nonspecific to specific incorporation in BSA can be estimated to exceed 500:1, assuming an average molecular weight of 100 for an amino acid and 66,200 for the protein. Therefore, the majority of ^{14}C labeling is at the nonspecific sites. As a consequence, a lesser ^{14}C-halothane incorporation in the presence of a competitor (e.g., isoflurane) is likely due to an excessive photolysis reaction of the competitor at the nonspecific sites, leaving fewer nonspecific sites for ^{14}C-halothane labeling. For the same reason, any differences in photolysis rates between the two optical enantiomers may contribute to the observed difference in the apparent K_I.

In a series of ^{19}F NMR studies (Dubois and Evers, 1992; Dubois et al., 1993), Dubois and co-workers investigated the binding of racemic isoflurane and several other fluorinated anesthetics to BSA. Our results on chemical shifts, X_b, and T_2 with racemic isoflurane were in good agreement with theirs. Although BSA plays no mechanistic role in general anesthesia, the low affinity of anesthetic binding to BSA may represent a general mode of anesthetic-protein interaction in vivo, where the anesthetics exchange rapidly among various molecular and cellular compartments. Therefore, in contrast to the static view of anesthetic agents bound to certain clefts in a crucial protein, general anesthesia may

reflect a dynamic process between anesthetics and proteins (Dilger et al., 1994). The dynamic association rate constant, k_{on}, is an indicator of the goodness of fit between an anesthetic and its protein target, whereas the dissociation rate constant, k_{off}, is a reflection of the strength of binding. Thus, although the effects of an anesthetic on a given protein are dependent on the equilibrium effective concentration (e.g., EC_{50}), the details of the anesthetic-protein interaction (comprised by the k_{on} and k_{off}) may be much more revealing. For example, the S(+) enantiomer could bind to the pore of a channel protein with longer duration (smaller k_{off}) to produce a stronger effect, even when the K_D appeared the same because of a slower association rate (smaller k_{on}). Differences in binding and dissociation rates have been suggested to be the basis of a unitary mechanism of action of ether, isoflurane, and propofol on ACh receptor channel conductance (Dilger et al., 1994). We have shown that such differences can also account for the observation of stereoselectivity in vitro, and may do so in vivo.

In conclusion, isoflurane binding to BSA is stereoselective. Stereoselectivity results from the differences in the rates at which the enantiomers bind to and dissociate from the high affinity binding sites in BSA.

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