Thermodynamics of anesthetic/protein interactions

Temperature studies on firefly luciferase

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ABSTRACT Firefly luciferase is a soluble enzyme which is unusually sensitive to general anesthetics. The inhibition of the highly purified enzyme by three inhalational and three alcohol general anesthetics has been studied as a function of temperature, in the range from 5 to 20°C. Inhibition constants K_i were determined at different temperatures, and van't Hoff plots of ln (K_i) versus reciprocal absolute temperature were found to be linear for all agents. Analysis of these plots gave values for the standard Gibbs free energy, enthalpy and entropy changes for transferring each anesthetic from water to the anesthetic-binding pocket on the protein. The most striking finding was that the enthalpy changes were much more negative for anesthetics binding to the protein than for binding to lipids or simple solvents. Furthermore, amongst the set of anesthetics studied, it was found that increasing potency correlated with favorable enthalpy rather than entropy changes. We discuss our results with respect to the molecular mechanisms underlying general anesthesia.

INTRODUCTION

What makes a protein particularly sensitive to general anesthetics? In view of the increasing evidence that anesthetics act directly on proteins to produce general anesthesia (1, 2), this has become an important question. This matter is easiest to study with a pure soluble protein, where any possible problems due to lipid involvement are absent. However, the functions of most soluble proteins which have been investigated have been found to be surprisingly insensitive to anesthetics (1). Fortunately, there are exceptions to this rule. The best studied of these is the enzyme firefly luciferase, which is sensitive to a wide range of different anesthetics at concentrations similar to those which induce general anesthesia in animals (3).

It is known from previous studies (3–7) that general anesthetics inhibit firefly luciferase by binding to a hydrophobic pocket which normally binds its natural substrate luciferin. Many other soluble proteins have substrate-binding pockets with considerable hydrophobic character; however, only a few of these appear to be sensitive to a wide range of general anesthetics. What is a mystery, therefore, is why the luciferase pocket binds so many general anesthetics so avidly, when hydrophobic pockets on other proteins do not. One constraint on possible solutions to this problem comes from a knowledge of the thermodynamics of anesthetic/protein interactions, an area which has been much neglected. In this paper, we describe our first experiments on the temperature dependence of anesthetic inhibition of a protein (firefly luciferase) and report the results of our determinations of the underlying thermodynamic parameters.

MATERIALS AND METHODS

Purification and assay of firefly luciferase

The luciferase enzyme from the North American firefly *Photinus pyralis* was purified using affinity chromatography (8); a pure crystalline protein was obtained (9), free of the natural substrate luciferin. The enzyme was stored as a stock solution in 0.4 M ammonium sulfate/1 mM EDTA solution (pH 7.8) at 4°C. Hepps [*N*-(2-hydroxyethyl)-piperazine-*N'*-(3-propane-sulfonic acid)], ATP (disodium salt, grade I), and D-luciferin were purchased from Sigma Chemical Co. (Poole, Dorset, UK). $MgSO_4$ (Analar grade), *n*-butanol, *n*-hexanol and *n*-heptanol (Analar grade) were obtained from BDH Ltd. (Poole, Dorset, UK). The volatile general anesthetics were obtained from the following sources: halothane from May & Baker Ltd. (Dagenham, Essex, UK), methoxyflurane (Penthrane®) from Abbott Laboratories Ltd. (Queenborough, Kent) and diethyl ether (AR grade) from Fisons Ltd. (Loughborough, Leicestershire, UK).

Firefly luciferase combines with its substrate luciferin in the presence of ATP, Mg^{2+}, and O_2 to give a photon of light. Luciferase activity and its inhibition by anesthetics over a range of temperatures were determined (see below) from the peak light output observed after rapidly injecting 2.5 ml of a buffered ATP solution into a reaction vial containing 5 ml of a buffered solution containing the luciferase enzyme, its natural substrate firefly luciferin, $MgSO_4$, and anesthetic (when appropriate). The buffer was 25 mM Hepps, titrated to pH 7.8 at 20°C with NaOH. (Hepps was chosen because of its favorable pK_a = 8.0 and its low temperature coefficient: buffer pH increased by only 0.2 units on cooling from 20°C to 5°C.) Final concentrations were 4 mM ATP, 6.67 mM $MgSO_4$, 1–45 μM luciferin, and 13 nM luciferase. Inhalational general anesthetics were added to the reaction vial as aliquots of saturated aqueous solutions.

Rapid-injection apparatus and temperature control

Light-emitting luciferase assays were performed using a specially constructed temperature-controlled rapid-injection apparatus. This allowed a known volume of solution in a glass syringe to be rapidly injected (by the action of a gas-driven piston) into a glass reaction vial (a standard 21-ml capacity scintillation vial) situated within a light-tight assay chamber. The solution was delivered to the vial through a polyvinylchloride (PVC) tube connected to the syringe via a large bore stainless steel needle (with a Luer-lock fitting) and to the vial via a curved stainless-steel tube passing through a rubber bung. The rubber bung provided both a light-tight seal to the assay chamber and mechanical support for the delivery tube, allowing the end of the tube to be reproducibly positioned with respect to the reaction vial. Light from the assay chamber passed via a shutter through a neutral density filter to a chamber housing a photomultiplier (No. 9558B; Thorn EMI Ltd., Ruislip, Middlesex, UK). The signal from the photomultiplier was taken to a variable-gain current-voltage converter, whose output was

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transiently stored on a digital storage oscilloscope. Permanent records were produced by sending data from the oscilloscope to a chart recorder.

Temperature was carefully controlled as follows. The temperature of the glass injection syringe, the assay chamber, and the connecting tubing was set and maintained using a water cooler and heater/pump (Grant Instruments Ltd., Cambridge, UK). The pump circulated water through Dural blocks housing the assay chamber and surrounding the injection syringe, as well as along a large rubber tube which enclosed the connecting PVC delivery tube. Numbered reaction vials were prepared containing the relevant solutions (excluding enzyme and volatile anesthetics). These were capped and then placed in a water bath, controlled by the same heater/cooler unit, and allowed to equilibrate to the set temperature. The temperature of a dummy vial containing assay buffer was monitored with a thermocouple and digital thermometer. Volatile agents were delivered to the vials as saturated solutions, using a micrometer-driven glass dispensing syringe placed inside a cooled Dural block. The anesthetic solution was added (using an L-shaped needle) below the surface of the solution in the vial, just prior to the assay. In all cases, the enzyme solution (10 μl) was the very last component to be added to the vial before ATP injection. The ATP solution was kept in a covered beaker in the water bath, and the beaker was removed briefly to allow the injection syringe to be loaded. The temperature of the ATP solution was measured with a mercury-in-glass thermometer. The system was calibrated by monitoring the temperature of an assay vial during an assay by means of a thermocouple. It was found that the measured temperature of the ATP solution was an accurate measure of the final assay temperature, with a small correction factor at the lowest temperatures.

Inhibition of luciferase by anesthetics

Anesthetic inhibition of the luciferase enzyme was analyzed in terms of a simple random binding model described previously (3, 5). Since anesthetics compete with luciferin for binding to the enzyme, it is convenient to introduce (3) a function $f(I)$, defined as the factor by which the apparent Michaelis constant for luciferin increases in the presence of inhibiting anesthetic at a free concentration $[I]$. It is easy to show (5) that

$$f(I) = (v_o/v_i) + ([Luc]/K_m)(v_o/v_i - 1), \quad (1)$$

where v_o is the control activity, v_i is the inhibited activity, K_m is the Michaelis constant for luciferin in the absence of inhibitor, and $[Luc]$ is the luciferin concentration.

Inhibition experiments were carried out at a concentration (1 μM) of luciferin far below the value of its Michaelis constant K_m (which varied from 15 ± 2 μM at 7°C to 26 ± 2 μM at 19°C). Under these conditions, it can be seen from Eq. 1 that the actual value of K_m has only a minor effect on the calculated values of $f(I)$. Nonetheless, the appropriate value of K_m was used in Eq. 1 to calculate all $f(I)$ values.

Inhibition by the small anesthetic molecules considered in this paper was consistent with two molecules of a given anesthetic being able to bind independently to the enzyme, each with the same dissociation (inhibition) constant K_i, but with only one molecule being necessary to cause inhibition (3–5). For this case, it is easy to show (3) that

$$f(I) = (1 + [I]/K_i)^2, \quad (2)$$

so that

$$K_i = [I]/\{\sqrt{f(I)} - 1\}. \quad (3)$$

For the determination of the inhibition constants K_i for the various anesthetics, the inhibited activities v_i were determined in duplicate at five different anesthetic concentrations $[I]$; each individual inhibitor assay was paired with a control assay, which gave the activity v_o in the absence of inhibitor. From each (v_i, v_o) data pair, an estimate of the function $f(I)$ was made using Eq. 1, and then an estimate of the inhibition constant K_i was made using Eq. 3. Finally, the mean value and standard error of the inhibition constant K_i at each temperature were calculated from the (typically 10) individual estimates of K_i using the method of weighted least squares. The weighting factors $w(I)$, derived by assuming a constant percentage error in the measurement of enzyme activity, were

$$w(I) = [I]^2(v_i/v_o)^2 f(I)/(K_i)^4. \quad (4)$$

Thermodynamics of anesthetic binding to luciferase

From the temperature dependence of the inhibition constant K_i for the inhibition of firefly luciferase by each anesthetic, changes in the standard Gibbs free energy $\Delta G^0_{wat\to luc}$, enthalpy $\Delta H^0_{wat\to luc}$ and entropy $\Delta S^0_{wat\to luc}$ were determined for the transfer of one mole of anesthetic from aqueous buffer to the luciferase enzyme at 20°C. The standard state used was 1 molar anesthetic. Data were analysed using linear van't Hoff plots of $\ln(K_i)$ versus T^{-1}, where T is the absolute temperature.

The three thermodynamic transfer parameters and the inhibition constant are related by the equations

$$\ln(K_i) = [\Delta G^0_{wat\to luc}]/(RT) = [\Delta H^0_{wat\to luc}]/(RT) - [\Delta S^0_{wat\to luc}]/R, \quad (5)$$

where R is the gas constant. We determined the constants c_1 and c_2 and their variance-covariance matrix in the equation

$$\ln(K_i) = c_1 + c_2 T^{-1}, \quad (6)$$

using the individual K_i determinations (typically ten at each of 3 to 5 different temperatures in the range 5 to 20°C) and the method of weighted least squares. The weighting factors for $\ln(K_i)$, derived under the same assumption as that for K_i (see Eq. 4), were

$$w(I) = [I]^2(v_i/v_o)^2 f(I)/(K_i)^2. \quad (7)$$

It follows (10) from Eqs. 5 and 6 that at $T_0 \equiv 293.15$ K (i.e., 20°C),

$$\Delta G^0_{wat\to luc} \pm \mathrm{SE} = (c_2 + c_1 T_0)R \pm R[T_0^2 \,\mathrm{Var}(c_1) + 2T_0 \,\mathrm{Cov}(c_1, c_2) + \mathrm{Var}(c_2)]^{1/2}, \quad (8a)$$

$$\Delta H^0_{wat\to luc} \pm \mathrm{SE} = c_2 R \pm R[\mathrm{Var}(c_2)]^{1/2}, \quad (8b)$$

and

$$\Delta S^0_{wat\to luc} \pm \mathrm{SE} = -c_1 R \pm R[\mathrm{Var}(c_1)]^{1/2}, \quad (8c)$$

where the variance and covariance terms are the diagonal and off-diagonal elements, respectively, of the variance-covariance matrix for c_1 and c_2.

Equations 8, *a–c*, were used to calculate the thermodynamic parameters and their standard errors for the transfer of anesthetics from the aqueous phase to the luciferase enzyme at 20°C.

RESULTS

Effects of temperature on the uninhibited reaction

The highly purified firefly luciferase enzyme was studied under conditions (4 mM ATP) in which its ATP-binding site was effectively saturated, placing the enzyme in a

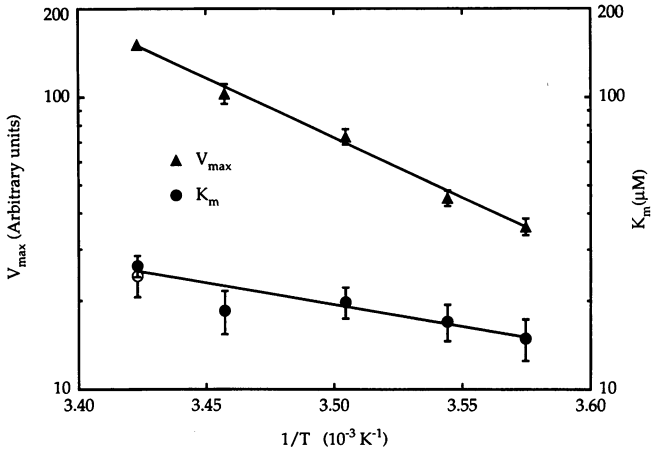

FIGURE 1 Temperature dependence of the uninhibited firefly luciferase activity. Values of the maximal activity V_{max} (▲) and the luciferin Michaelis constant K_m (●, ○) are plotted (on logarithmic scales) against the reciprocal of the absolute temperature. V_{max} values were corrected for the decay which occurred during the course of the experiment. At 19°C, the closed (●) and open (○) symbols for K_m refer to values measured at the start and finish of the experiment, respectively. V_{max} and K_m values were determined at each temperature from double reciprocal plots of (activity)$^{-1}$ versus (luciferin concentration)$^{-1}$, using the method of weighted least squares with weighting factors proportional to the squares of the activities (3). The error bars are standard errors; where not shown, these are smaller than the symbols. The straight lines in the figure were calculated using the method of weighted least squares, with weighting factors proportional to the reciprocal of the squares of the standard errors.

form known to be highly sensitive to inhibition by a wide range of anesthetic agents (3–6). The effects of temperature upon the uninhibited enzyme were first determined. As reported previously (11), activity was found (not shown) to increase with rising temperature from 6°C up to ~25°C, after which it decreased, presumably due to thermal inactivation of the enzyme. To be certain that our observed effects would not be complicated by thermal inactivation, we chose to work in the temperature range between ~5 and 20°C.

Fig. 1 shows the behavior of V_{max} and the K_m for luciferin as a function of reciprocal temperature (K^{-1}) over the temperature range 7 to 19°C. It can be seen that both parameters increased with rising temperature, but the effect on V_{max} (a 4.2-fold variation) was about three times greater than the effect on K_m (only a 1.7-fold variation). From the slope of the line for V_{max}, the activation energy for the catalytic steps was calculated to be (mean ± SEM) E_{act} = 78 ± 2 kJ mol^{-1}, which is similar to the value of 75 kJ mol^{-1} reported by McElroy and Seliger (11) under unspecified concentrations of ATP and luciferin.

Competitive nature and stoichiometry of anesthetic inhibition

Inhibition of firefly luciferase at room temperature by a wide range of anesthetic molecules is competitive with respect to luciferin (3–7). The same was found to be true at low temperatures. This is illustrated for halothane by the data in Fig. 2, which is a double reciprocal plot of (activity)$^{-1}$ versus (luciferin concentration)$^{-1}$ at different concentrations of halothane at 6°C. The common interception of the lines on the ordinate axis of Fig. 2 indicates that V_{max} is unaffected by anesthetic, i.e., that anesthetics compete with luciferin for binding to the enzyme.

In addition, for all anesthetics considered in this paper, inhibition was consistent with a simple scheme (3–5) in which two molecules of a given anesthetic can bind independently to the enzyme, each with the same dissociation (inhibition) constant K_i, but only one bound molecule is necessary to cause inhibition. This scheme predicts (see Eq. 2) that the square root of $f(I)$ (where $f(I)$ is defined as the factor by which the apparent Michaelis constant for luciferin increases in the presence of a concentration $[I]$ of inhibiting anesthetic) is a linear function of $[I]$. This behavior was observed for all anesthetics used here and is illustrated in Fig. 3 for methoxyflurane, at five different temperatures.

Effects of temperature on anesthetic inhibition of luciferase

Anesthetic inhibition constants K_i were determined over a range of temperatures. At each of 3 to 5 temperatures and at each of 5 different anesthetic concentrations, determination of the mean ± SEM of K_i involved (typically) 10 measurements of enzyme activity in the pres-

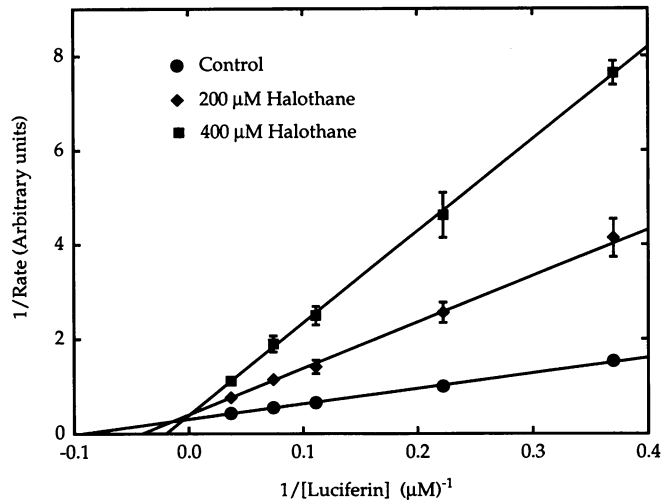

FIGURE 2 Anesthetic inhibition of firefly luciferase is competitive with respect to luciferin at low temperatures. The reciprocal of the luciferase activity at 6°C is plotted against the reciprocal of the luciferin concentration at different concentrations of halothane: 0 (●); 200 (◆); 400 μM (■). All points but one are means of triplicate determinations. The error bars are standard errors; where not shown, these are smaller than the symbols. The straight lines were calculated using the method of weighted least squares, with weighting factors proportional to the reciprocal of the squares of the standard errors.

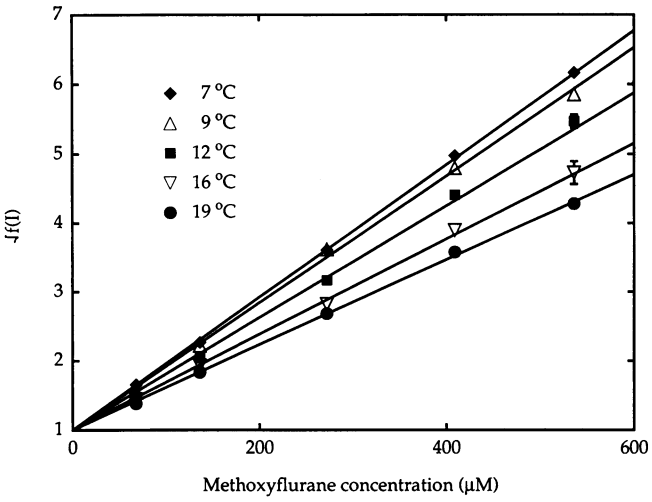

FIGURE 3 Two molecules of anesthetic interact with firefly luciferase at all temperatures and bind with greater affinity at lower temperatures. The square root of the function $f(I)$ (≡ the factor by which the K_m for luciferin increases in the presence of inhibitor at a concentration $[I]$) is plotted against the methoxyflurane concentration at the following temperatures: 7 (♦); 9 (△); 12 (■); 16 (▽); 19°C (●). The values of $f(I)$ were calculated from duplicate measurements of both control and inhibited activities, using Eq. 1. The error bars are standard errors; where not shown, these are smaller than the symbols. Each straight line was drawn according to the equation (see Eq. 2) $\sqrt{f(I)} = (1 + [I]/K_i)$, using the mean value of 10 separate determinations of the inhibition (dissociation) constant K_i.

ence and 10 measurements in the absence of anesthetic. According to the simple binding scheme, these inhibition constants represent the equilibrium dissociation constants for anesthetics binding to the luciferin-binding pocket of the enzyme (5). For all agents used, K_i was found to increase with increasing temperature, indicating that anesthetic binding decreases with increasing temperature. This can be seen in Fig. 4 for the inhalational general anesthetics and in Fig. 5 for the alcohol general anesthetics, where the data are plotted as van't Hoff plots of ln (K_i) versus reciprocal absolute temperature.

The six van't Hoff plots shown in Figs. 4–5 were further analyzed to obtain values for the changes in standard Gibbs free energies, enthalpies and entropies for transfer of anesthetics from aqueous buffer to the anesthetic-binding pocket on the luciferase enzyme. These values are listed in Table 1. Since the van't Hoff plots were linear, the enthalpy and entropy changes are constant over the entire temperature range considered (from ~5 to 20°C). The free energy changes listed, however, refer only to 20°C; values at other temperatures can be calculated using Eq. 5.

DISCUSSION

The temperature dependence of the binding of six anesthetics to the anesthetic-sensitive (high ATP) form of firefly luciferase was studied over a temperature range (~5 to 20°C) in which the uninhibited enzyme behaved reasonably (Fig. 1) and did not suffer from thermal inactivation. Inhibition was competitive (for example, see Fig. 2) with respect to the natural substrate luciferin at all temperatures, and two molecules of each anesthetic could bind to the enzyme (Fig. 3). Van't Hoff plots (Figs. 4–5) of the natural logarithm of the inhibition constants K_i versus reciprocal absolute temperature were linear, and quantitative analysis of these plots yielded values for changes in the standard Gibbs free energies, enthalpies and entropies for transfer of anesthetic molecules from water to the anesthetic-binding site (see Table 1).

The values of the standard Gibbs free energy changes $\Delta G^0_{wat \to luc}$ at 20°C are quite precise, the largest standard error being less than 0.6% of the mean. They show reasonable agreement with estimates calculated (Eq. 5) from earlier (3–4), less precise K_i determinations at room temperature and lower (2 mM) ATP concentrations.

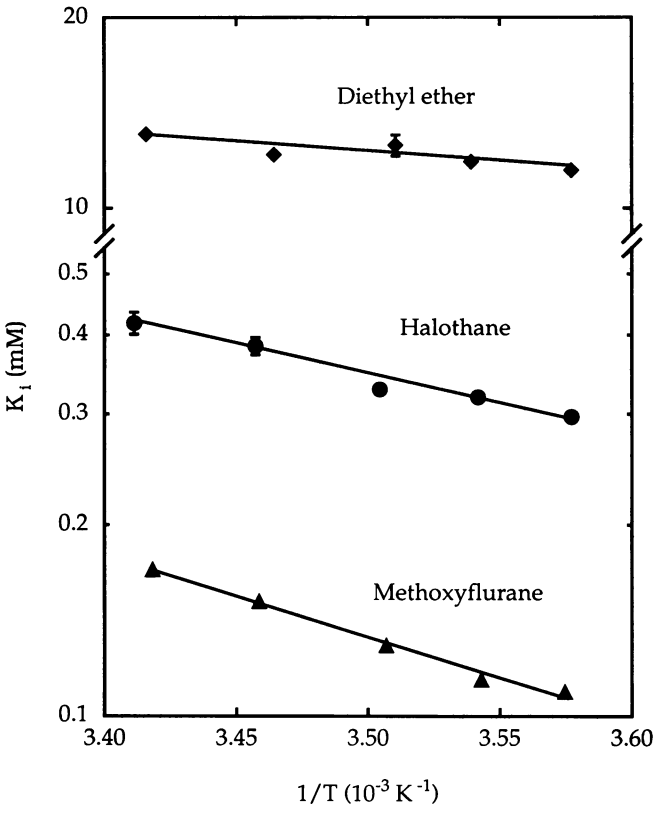

FIGURE 4 Van't Hoff plots for the inhalational general anesthetics diethyl ether (♦), halothane (●), and methoxyflurane (▲). The mean inhibition constants K_i (on logarithmic scales) are plotted against the reciprocal of the absolute temperature. Each mean K_i value is the average of (typically) 10 separate K_i determinations. The error bars are standard errors; where not shown, these are smaller than the symbols. The straight lines were drawn according to the method of weighted least squares, using (typically) 50 separate estimates of ln (K_i) and weights given by Eq. 7.

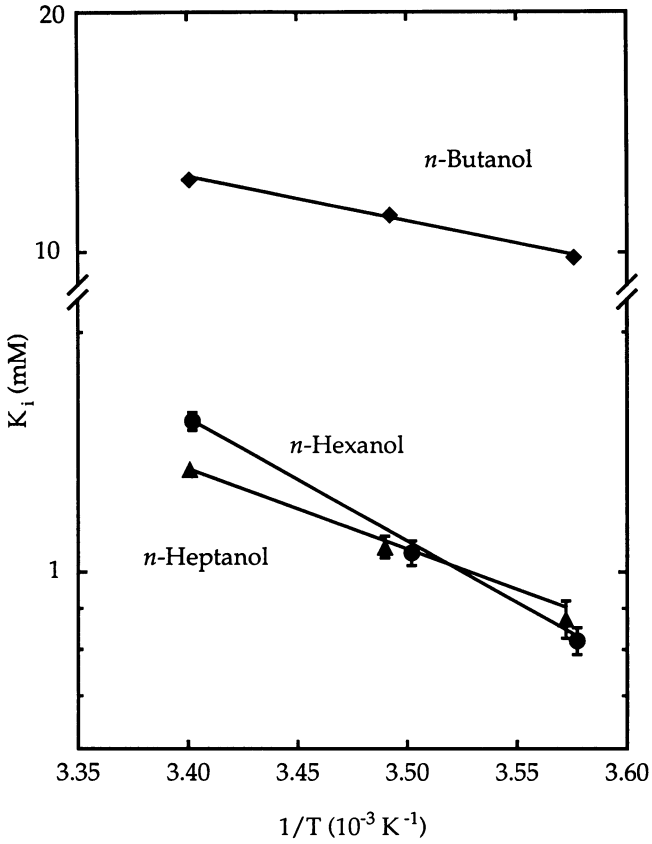

FIGURE 5 Van't Hoff plots for the alcohol general anesthetics *n*-butanol (♦), *n*-hexanol (●), and *n*-heptanol (▲). The mean inhibition constants K_i (on logarithmic scales) are plotted against the reciprocal of the absolute temperature. Each mean K_i value is the average of (typically) 10 separate K_i determinations. The error bars are standard errors; where not shown, these are smaller than the symbols. The straight lines were drawn according to the method of weighted least squares, using (typically) 30 separate estimates of ln (K_i) and weights given by Eq. 7.

Our values of transfer enthalpies and entropies ($\Delta H^0_{wat\to luc}$ and $\Delta S^0_{wat\to luc}$, respectively) from the aqueous phase to the anesthetic-binding pocket are listed in Table 1. Although enthalpies and entropies of transfer for inhalational agents from the gas phase to firefly luciferase were reported in an early study by Ueda and Kamaya (12), their values are suspect for the following reasons. First, it was not known until a decade after their study was carried out that anesthetics inhibit luciferase by competing with luciferin (3), and unfortunately they used crude extracts of firefly lanterns containing unknown (and possibly variable) concentrations of luciferin. Since the binding of luciferin, and its variation with temperature, can substantially affect the binding of anesthetics, it is not now possible to accurately reanalyze their data. Furthermore, they interpreted their data using an old kinetic scheme inherited from earlier workers, in which anesthetics only interact with a thermally inactivated form of the enzyme. This unrealistic scheme (see discussion in reference 1) led them to report extremely large decreases in both enthalpy and entropy on binding of anesthetics.

Many workers (for a review, see reference 13) have attempted to use values of enthalpy and entropy changes such as those in Table 1 to determine whether binding is "enthalpy-driven" or "entropy-driven". Since $\Delta G^0 = \Delta H^0 - T\Delta S^0$, they reason that the total free energy change ΔG^0 can be subdivided into an enthalpy contribution (ΔH^0) and an entropy contribution ($-T\Delta S^0$). However, although the enthalpy changes are independent of the choice of a standard state, the same is not true for the free energy and entropy changes. One might be tempted, for example, to look at the results for *n*-butanol in Table 1 and say that its binding to luciferase is almost entirely "enthalpy-driven" (since $\Delta G^0 \approx \Delta H^0 = -10.7$ kJ mol^{-1} and $-T\Delta S^0 \approx 0$ kJ mol^{-1}). However, if we had arbitrarily chosen 100 M anesthetic (rather than 1 M) as the standard state, the enthalpy change would remain as $\Delta H^0 = -10.7$ kJ mol^{-1} but ΔG^0 and $-T\Delta S^0$ would both decrease by $RT\ln(100) = 11.2$ kJ mol^{-1}, so that $\Delta G^0 = -21.9$ kJ mol^{-1}, $\Delta H^0 = -10.7$ kJ mol^{-1} and $-T\Delta S^0 = -11.2$ kJ mol^{-1}. We might then conclude that the binding of butanol was equally driven by enthalpy and entropy changes!

The above example illustrates the dangers of discussing free energy and entropy changes for ligand binding in absolute terms. (Enthalpy changes, as already mentioned, do not suffer from standard state difficulties, and we shall compare and discuss these for different transfer processes in the following sections.) However, one *can* consider incremental $\Delta(\Delta G^0)$ and $\Delta(\Delta S^0)$ changes [and, of course, $\Delta(\Delta H^0)$ changes] between different ligands without standard state problems. In Fig. 6, we have plotted for our luciferase data both $-T\Delta S^0_{wat\to luc}$ and $\Delta H^0_{wat\to luc}$ versus $\Delta G^0_{wat\to luc}$ at 12°C (roughly the mid-point temperature). The slopes of the solid least squares lines drawn through the data points, when compared to the slope of the dashed line assuming the theoret-

TABLE 1 Changes in standard Gibbs free energy, enthalpy and entropy for transfer of anesthetics from aqueous buffer to the luciferase enzyme at 20°C

Anesthetic	n_{obs}	$\Delta G^0_{wat\to luc}$	$\Delta H^0_{wat\to luc}$	$\Delta S^0_{wat\to luc}$
		kJ mol^{-1}	*kJ mol^{-1}*	*J mol^{-1} K^{-1}*
Halothane	50	−18.93 ± 0.05	−18.1 ± 1.6	2.8 ± 5.7
Methoxyflurane	50	−21.10 ± 0.03	−24.5 ± 1.0	−11.7 ± 3.5
Diethyl ether	50	−10.56 ± 0.06	−5.9 ± 1.8	16.0 ± 6.4
n-Butanol	29	−10.72 ± 0.04	−10.7 ± 1.1	0.05 ± 3.88
n-Hexanol	30	−15.85 ± 0.08	−29.5 ± 2.3	−46.7 ± 8.1
n-Heptanol	30	−16.17 ± 0.08	−19.3 ± 2.5	−10.7 ± 8.7

The standard state is 1 M anesthetic. The errors are standard errors based on n_{obs} measurements of inhibition constants K_i over the temperature range from ~5 to 20°C.

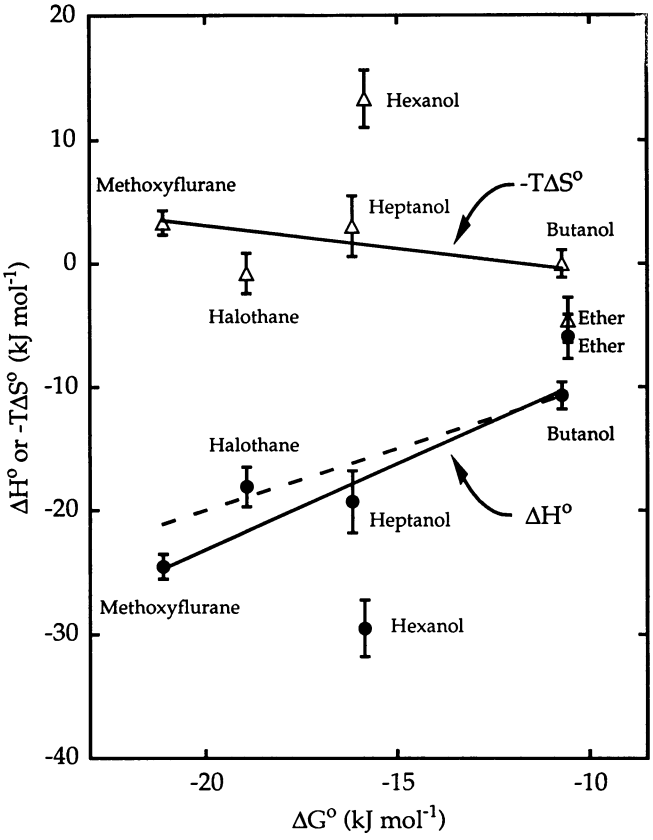

FIGURE 6 Amongst the set of anesthetics studied, increasing potency for inhibiting luciferase correlates with favorable enthalpy rather than entropy changes. Values of $-T\Delta S^0_{wat\rightarrow luc}$ (△) and $\Delta H^0_{wat\rightarrow luc}$ (●) are plotted against $\Delta G^0_{wat\rightarrow luc}$, using data from Table 1 and the mid-point temperature T = 285.15 K (i.e., 12°C). The error bars are standard errors. The solid straight lines were drawn according to the method of weighted least squares, using weighting factors proportional to the reciprocal of the squares of the standard errors of the ordinate values; the slopes (±SEM) are −0.38 ± 0.39 for the upper ($-T\Delta S^0$) line and 1.38 ± 0.40 for the lower (ΔH^0) line. The dashed line is the theoretical relationship $\Delta H^0 \equiv \Delta G^0$.

ical relationship $\Delta H^0 \equiv \Delta G^0$, show that the incremental changes $\Delta(\Delta G^0)$ in binding free energy are largely accounted for by changes $\Delta(\Delta H^0)$ in enthalpy rather than changes $\Delta(\Delta S^0)$ in entropy. In other words, although we cannot deduce from our data the relative contributions of entropy and enthalpy to the absolute binding constant of a given anesthetic, we can conclude that, on average, increasing potency correlates with favorable changes in enthalpy, with entropy changes, if anything, tending to oppose enhanced binding.

Comparison with solvents and lipids

The most striking result of these experiments is that for all general anesthetics studied, the enthalpies of transfer (Table 1) from water to the anesthetic-binding site on firefly luciferase are algebraically much more negative (by ≥18 kJ mol^{-1}) than would be predicted from transfer into simple solvents or lipid bilayers. This can be seen from Table 2, where we have listed the corresponding enthalpies of transfer of the same anesthetics from water to cholesterol-containing lipid bilayers, olive oil and alcohols (see also reference 14). It is clear from this table that either no significant heat is released, or heat is actually absorbed, when anesthetics bind to lipids or solvents from water, whereas heat is released when anesthetics bind to luciferase from water.

It is tempting to suppose that these large excess (relative to solvents and lipid bilayers) negative enthalpies of transfer from water to luciferase (see Table 2) underlie the unusual sensitivity of this enzyme to anesthetics. However, as the example in the last section demonstrated, such a conclusion cannot be reliably drawn from our current data. Nonetheless, this is an attractive hypothesis, and future work, on the temperature dependence of anesthetic binding to the insensitive form of firefly luciferase which exists at low ATP concentrations (5), should test this idea and help to clarify why some proteins are sensitive to general anesthetics while most are not.

Temperature dependence of general anesthesia

The potency of a general anesthetic agent for inducing general anesthesia in a given animal is a function of both temperature and pressure. While the pressure dependence has long been thought to provide clues as to how

TABLE 2 **Enthalpies of transfer of general anesthetics from the aqueous phase to firefly luciferase, olive oil, lipids and *n*-alcohols**

	$\Delta H^0_{water\rightarrow site}$ (kJ mol^{-1})			
Anesthetic	Luciferase*	Olive Oil‡	Lipid§	*n*-Alcohol‖
Halothane	−18 ± 2	+4 ± 5	+11 ± 1	—
Methoxyflurane	−24 ± 1	−6 ± 4	+1 ± 2	—
Diethyl ether	−6 ± 2	+13 ± 2	—	—
n-Butanol	−11 ± 1	—	—	+9.3
n-Hexanol	−30 ± 2	—	—	+6.5
n-Heptanol	−19 ± 2	—	—	+5.3

* Luciferase values (±SEM) are from Table 1. All other values (±SEM) were calculated from slopes of linear regions (always including 25°C) of appropriate van't Hoff plots, using unweighted linear regressions on data from the literature (see below). The olive oil and lipid values were calculated from gas phase measurements using data (20, 22) on the partitioning of the anesthetic gases between water and gas phases as a function of temperature. ‡Bunsen coefficients were calculated from Ostwald oil/gas partition coefficients (20). §Lipid/water partition coefficients were calculated as the ratios of Bunsen coefficients (22) for lipid bilayers (egg lecithin/phosphatidic acid/cholesterol) and for water. ‖Standard enthalpies of solution at 25°C, taken from reference 24. (—) Relevant data or values are not available.

general anesthetics act (15–18), less attention has been paid to the temperature dependence. This neglect might be justified on the grounds that animals, especially homeotherms, have evolved physiological and behavioral strategies for maintaining constant internal temperatures. In addition, reduction of body temperature per se might contribute towards the anesthetic endpoint (19).

Nonetheless, although a priori it is perhaps naive to analyze the effects of pressure and temperature on whole animal anesthetic potencies in the same terms as for simple chemical reactions, it is interesting to perform the exercise (1). If for simplicity we assume that temperature is primarily affecting the binding of anesthetics to critical sites in the central nervous system, and that anesthesia occurs when some constant fraction of sites is occupied by one or more anesthetic molecules, it is easy to show that $\ln(EC_{50}) = \Delta H/RT + \text{constant}$, where EC_{50} is the concentration of anesthetic which anesthetizes 50% of a population of animals at an absolute temperature T, and ΔH is the apparent enthalpy change for transferring an anesthetic from any phase (here water) to the unknown animal target sites. This means that one can calculate ΔH from a van't Hoff plot of $\ln(EC_{50})$ versus T^{-1}.

We have performed such an analysis for the three inhalational agents used for the luciferase study for both a "warm-blooded" animal (the dog) and a "cold-blooded" animal (the goldfish). For the dog, using gaseous minimum alveolar concentrations (MAC) in the temperature range 26 to 43°C (19–21) and converting these to aqueous EC_{50} values (20, 22), the apparent ΔH (mean ± SEM) values are -11 ± 3 kJ mol^{-1} for halothane, -22 ± 8 kJ mol^{-1} for methoxyflurane, and 8 ± 10 kJ mol^{-1} for diethyl ether. For the goldfish, using directly determined aqueous EC_{50} values (23) at 10, 20, and 30°C, the apparent ΔH values (errors not given since there were only three data points for each van't Hoff plot) are -14 kJ mol^{-1} for halothane, -14 kJ mol^{-1} for methoxyflurane, and 11 kJ mol^{-1} for diethyl ether.

In view of the simplifying assumptions made, it is interesting that the apparent enthalpy changes for a given agent are comparable (indeed equivalent within errors) for the two different animals. Furthermore, for halothane and methoxyflurane the enthalpy changes are (as for luciferase) more negative for transfer of the anesthetics to the animal target sites than for transfer to lipid bilayers. (Most lipid theories of general anesthesia postulate a disordering of the membrane by anesthetics, which then interferes with protein function. Such disordering would be mimicked by increasing temperature, thus making the animal enthalpy changes more positive (not more negative as observed) than those for lipid partitioning.) Thus these results are consistent with, but certainly do not prove, the idea that the primary animal target sites for general anesthesia are proteins whose sensitivity to anesthetics derives from the same type of mechanism underlying the sensitivity of firefly luciferase.

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