Is There a Specific Receptor for Anesthetics? Contrary Effects of Alcohols and Fatty Acids on Phase Transition and Bioluminescence of Firefly Luciferase

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ABSTRACT Firefly luciferase emits a burst of light when mixed with ATP and luciferin (L) in the presence of oxygen. This study compared the effects of long-chain *n*-alcohols (1-decanol to 1-octadecanol) and fatty acids (decanoic to octadecanoic acids) on firefly luciferase. Fatty acids were stronger inhibitors of firefly luciferase than *n*-alcohols. Myristyl alcohol inhibited the light intensity by 50% (IC_{50}) at 13.6 μM, whereas the IC_{50} of myristic acid was 0.68 μM. According to the Meyer-Overton rule, fatty acids are ~12,000-fold stronger inhibitors than corresponding alcohols. The Lineweaver-Burk plot showed that myristic acid inhibited firefly luciferase in competition with luciferin, whereas myristyl alcohol inhibited it noncompetitively. The differential scanning calorimetry (DSC) showed that an irreversible thermal transition occurred at ~39°C with a transition ΔH_{cal} of 1.57 cal g^{-1}. The ligand effects on the transition were evaluated by the temperature where the irreversible change is half completed. Alcohols decreased whereas fatty acids increased the thermal transition temperature of firefly luciferase. Koshland's transition-state theory (*Science.* 1963. 142:1533–1541) states that ligands that bind to the substrate-recognition sites induce the enzyme at a transition state, which is more stabilized than the native state against thermal perturbation. The long-chain fatty acids bound to the luciferin recognition site and stabilized the protein conformation at the transition state, which resisted thermal denaturation. Eyring's unfolding theory (*Science.* 1966. 154:1609–1613) postulates that anesthetics and alcohols bind nonspecifically to interfacial areas of proteins and reversibly unfold the conformation. The present results showed that alcohols do not compete with luciferin and inhibit firefly luciferase nonspecifically by unfolding the protein. Fatty acids are receptor binders and stabilize the protein conformation at the transition state.

INTRODUCTION

Ample reports showed that anesthetics modify the function of ligand-gated ion channels, but the exact mechanisms and how they affect these channels remain unknown. Channels are highly organized protein-lipid complexes. Because anesthetic potencies approximately correlate to oil/water partition coefficients, lipid parts of cell membranes have been favored for the primary action site of anesthetics. Recently, the general opinion on anesthesia mechanisms changed from lipid theories to protein theories, which postulate direct anesthetic-protein interactions. A fundamental issue in the protein theories of anesthesia mechanisms is whether anesthetics act by binding to specific functional sites of proteins or by nonspecific allosteric conformational changes of proteins.

Because it is difficult to isolate lipid-free channel proteins in a functional state, model proteins have been employed. Firefly luciferase is uniquely fitted for this study, because the enzyme can be purified in lipid-free crystalline form, and the light intensity is highly sensitive to anesthetics and alcohols (Ueda, 1965; Franks and Lieb, 1984). Firefly luciferase emits a flash of light when substrates ATP and luciferin (L) are mixed in the presence of oxygen (McElroy and Seliger, 1962; DeLuca and McElroy, 1974):

$$\text{FFL} + \text{Luciferin} + \text{ATP} \rightleftarrows \text{FFL} \cdot \text{LuciferylAMP} + \text{PP}_i \quad (1)$$

$$\text{FFL}\cdot\text{LuciferylAMP} + \text{O}_2 \rightarrow \text{FFL} \cdot \text{OxyluciferylAMP} + \text{CO}_2 + \text{Light} \quad (2)$$

$$\text{Oxyluciferin} + \text{FFL} \rightleftarrows \text{FFL} \cdot \text{Oxyluciferin} \quad (3)$$

where FFL is firefly luciferase and PP_i is pyrophosphate.

We (Ueda and Kamaya, 1973) reported that anesthetics inhibited firefly luciferase allosterically by nonspecific conformational change of firefly luciferase. The anesthetic interaction was accompanied by a positive ΔH of ~80 kcal mol^{-1}. Dickinson et al. (1993) argued that the ΔH of anesthetic-luciferase interaction is about 5 kcal mol^{-1}. Their small negative ΔH represents the change in the energy level of volatile anesthetics (100–200 D) when they are transferred from water to the protein. Our large positive ΔH is the change in the energy level of firefly luciferase (62,000 D) when the protein is transferred from water to anesthetic solution.

Franks and Lieb (1984) reported that anesthetics and alcohols inhibited the enzyme in competition with the substrate luciferin. Although their article is widely quoted as evidence for the specificity of anesthetic action, it is the

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only report that anesthetics and alcohols inhibit enzymes in competition with substrates. All others concluded that the mechanism involved is allosteric inhibition. The reason for this unusual result may be that Franks and Lieb (1984) used initial flash intensity to evaluate the inhibition mode. The rapid reaction kinetics (Hiromi, 1979) designates the initial flash in a multiple reaction sequence as a pre-steady-state burst. Because inhibition kinetics must be analyzed under a steady-state condition, the present study used the steady-state light intensity to evaluate the inhibitor kinetics. It will be shown that alcohols do not compete with luciferin.

DeLuca (1969) reported that anilinonaphthalene sulfonate and toluidinonaphthalene sulfonate inhibit firefly luciferase in competition with luciferin. We (Chiou and Ueda, 1994; Ueda and Suzuki, 1998) found that myristic acid is a stronger inhibitor of firefly luciferase than ethanol or anesthetics and showed by differential scanning calorimetry that these chromophores and myristic acid increased the thermal transition temperature of firefly luciferase. In contrast, volatile anesthetics (Ueda et al., 1994) and ethanol (Chiou and Ueda, 1994) decreased the transition temperature. The inhibition modes of anesthetics and alcohols must be different from those of luciferin competitors. The present study compared the actions of long-chain fatty acids (decanoic to octadecanoic acids) with those of the same alkyl-chain lengths of *n*-alcohols (1-decanol to 1-octadecanol) on firefly luciferase.

EXPERIMENTAL PROCEDURE

Crystalline firefly luciferase from *Photinus pyralis*, sodium salt of D-luciferin, ATP, glycylglycine, 1-alkanols and sodium salts of long-chain fatty acids with alkyl-carbon chain lengths between 10 and 18, and arachidonic acid were obtained from Sigma (St. Louis, MO). Bioluminescence of firefly luciferase was measured by a pneumatically driven Durrum model D-110 stopped-flow apparatus (Sunnyvale, CA). The photomultiplier output was monitored by a Nicolet 310 digital recording oscilloscope (Madison, WI) and downloaded onto floppy disks. The firefly luciferase mixture contained luciferase and luciferin in 100 mM glycylglycine buffer (pH 7.8). The ATP mixture contained Na_2ATP and $MgSO_4$ in the glycylglycine buffer. Equal amounts (1.0 ml) of each solution were mixed. The final concentrations were 65 μM luciferin, 3 mM ATP, 6 mM $MgSO_4$, and 3 μg ml^{-1} firefly luciferase. The sample temperature was maintained at 18 $\pm$ 0.5°C. The alcohols and fatty acids were dissolved in a minimum amount of methanol and added to each solution. Control studies, without the ligands, confirmed that the added methanol did not affect the light intensity.

According to the rapid reaction kinetics, the light output curve was integrated, and the slope of the linear portion was used to estimate the reaction velocity. A steady-state condition can also be obtained by decreasing the reaction rate. The initial reaction of the light emission is freely reversible. The addition of pyrophosphate to the reaction mixture decelerates the reaction rates, and the light intensity is maintained at a higher level for a time period proportional to the added amount (McElroy and Seliger, 1962; Ueda et al., 1994). The Lineweaver-Burk plots were constructed by adding pyrophosphate to eliminate the initial peak. Firefly luciferase was dissolved in 100 mM glycylglycine buffer at 10 μg ml^{-1}, to which 3 mM ATP, 6 mM $MgSO_4$, and 10 mM pyrophosphate were added in a total volume of 310 μl to obtain steady-state light intensity. The 3 mM luciferin in the glycylglycine solution was added to the reaction mixture by an infusion pump at a constant rate of 0.278 μl s^{-1} (50 nmol min^{-1}) over 60 s. The dilution effect of the infusion was corrected by a computer.

Thermal phase transition was measured by a MicroCal MC2 differential scanning calorimeter (Northampton, MA). The reaction chamber (1.1918 cm^3) was filled with firefly luciferase dissolved in 0.5 M glycylglycine buffer (pH 7.8) at 3 mg ml^{-1} solvent. The glycylglycine concentration was higher than that of the light intensity study, because higher buffer concentration is required to solvate this highly hydrophobic protein at 3 mg ml^{-1}. The DSC thermograms were analyzed with Origin software (MicroCal).

We found that the firefly luciferase solution suddenly becomes turbid at the transition temperature. The irreversible transition was measured by transmittance of firefly luciferase solution at 400 nm by a Perkin-Elmer 554 UV-visible spectrophotometer (Norwalk, CT) equipped with a programmable digital linear temperature controller and a Peltier heat exchanger. The scan rate was 1.0°C min^{-1}. Firefly luciferase was dissolved in 100 mM glycylglycine buffer (pH 7.8) at 0.25 mg ml^{-1}. The firefly luciferase and glycylglycine concentrations were lower than in the DSC study because reproducible data can be obtained at lower firefly luciferase concentration. The actual temperature in the cuvette was monitored with a DigiTech model 5810 thermometer (United Systems, Dayton, OH) and a filament thermistor inserted into the cuvette with 0.01°C resolution. The transmittance data and temperature output were recorded in the Nicolet 310 digital recording oscilloscope and were downloaded onto floppy disks.

RESULTS AND DISCUSSION

Fig. 1 shows the dose-response curves of lauric acid and lauryl alcohol on the steady-state light intensity of firefly luciferase for comparison. The sigmoidal curves were drawn according to the Boltzmann equation. The data fit the theoretical curve rather well. The effects of C(10–18) acids and C(10–18) alcohols are summarized in Fig. 2, and the IC_{50} values (the ligand concentration that inhibits 50% light intensity) are shown in Table 1. Alcohols are weaker inhibitors of firefly luciferase than corresponding fatty acids. The IC_{50} value of myristyl alcohol was 13.6 μM, and that of myristic acid was 0.68 μM. The oil/water partition coefficients are 199 for myristic acid and 120,000 for myristyl alcohol (Hansch and Clayton, 1973). According to the Meyer-Overton rule, the anesthetic potency of myristyl alcohol should exceed that of myristic acid by 600-fold. Contrary to the rule, myristic acid was ~20 times more potent than myristyl alcohol. Hence myristic acid is

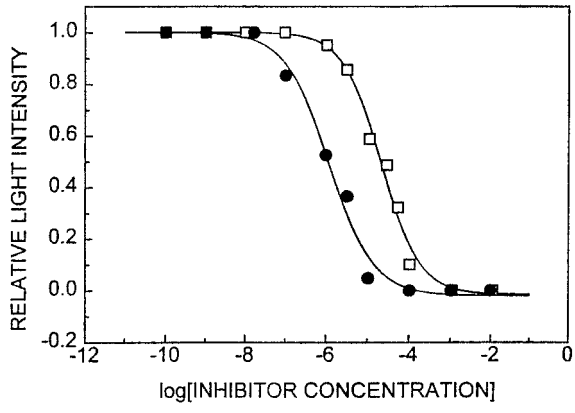

FIGURE 1 Dose-response curves of C12-alcohol (lauryl alcohol) and C12-acid (lauric acid). ●, C12-alcohol; □, C12-acid. Each point is the average of three determinations; the standard errors are within the symbol. The light intensities are measured by the slope of the integral of the stopped-flow tracing.

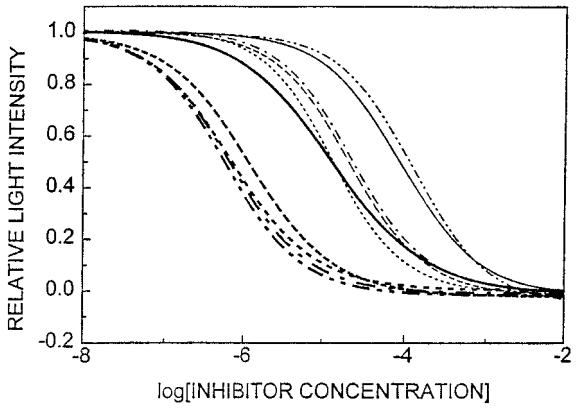

FIGURE 2 Dose-response curves of C10-C18 *n*-alcohols and fatty acids. Thick lines are fatty acids and thin lines are *n*-alcohols. Solid lines, C(10)s; dashes, C(12)s; dots, (C14)s; dash-dot-dash, C(16)s; dash-dot-dot-dash, C(18)s. Notice that myristyl alcohol shows maximum potency in the *n*-alcohol series. The potencies of fatty acids increased monotonously with the length of carbon chains. The dose-response curves of alcohols are steeper than those of fatty acids.

~12,000 (20 × 600) times stronger than myristyl alcohol when compared to the expected potency. Similar relationships are observed with other long-chain *n*-alcohols and corresponding fatty acids (Table 1). Furthermore, we found that arachidonic acid was also a strong inhibitor with IC_{50} of 0.62 μM. Notice that the rank order of the inhibitory effects of alcohols was highest with myristyl alcohol. Apparently, spatial restraint exists for alcohol adsorption. Fatty acids increased their potency monotonously with the increase in the carbon-chain lengths.

Dose-response curves of alcohols are clearly steeper than those of fatty acids (Figs. 1 and 2). The Hill numbers, n_H, of fatty acids were less than 1, whereas those of alcohols were more than 1 (Table 1). Although the difference appears to be small, the Hill number does not represent the actual number of ligand molecules that bind to the host molecule. This is because the definition of n_H assumes highly cooperative interaction between the enzyme E and *n* molecules of ligand L, where only one EL_n is produced:

$$E + nL \rightleftarrows EL_n \quad (4)$$

The intermediates, EL_1, EL_2, . . . , EL_{n-1}, are not counted. Therefore, n_H above one means that more than two ligand molecules bind to the enzyme. The Hill number is usually less than 2, and rarely exceeds 3. The actual maximum binding number B_{max} is much larger, by at least one order of magnitude. Our NMR study (Yoshida et al., 1997) on halothane binding to bovine serum albumin (BSA) showed a B_{max} of 34.5 at 37°C, and differential titration calorimetry (Ueda and Yamanaka, 1997) showed 13.2 for chloroform binding to BSA at 25°C. For this reason, the Hill number is often referred to as the cooperativity parameter. Alcohols interact cooperatively with multiple sites, whereas the binding sites for long-chain fatty acids are limited.

Under the steady-state condition, the Lineweaver-Burk plots of myristic acid showed competitive inhibition (Fig. 3), with $K_D = 0.53$ μM. Myristyl alcohol, however, showed noncompetitive inhibition, with $K_D = 20.5$ μM (Fig. 4). The notion (Franks and Lieb, 1984) that anesthetics and alcohols compete with luciferin may not hold because it was calculated by the effect on the initial pre-steady-state peak. Analysis of the inhibition mode in firefly luciferase by the Lineweaver-Burk plot requires caution because firefly luciferase light emission is a multistage reaction with three substrates, luciferin, ATP, and O_2, at different stages and two product inhibitions by pyrophosphate (initial stage) and oxyluciferin (final stage). The Lineweaver-Burk plot calcu-

TABLE 1 Effects of 1-alkanols and long-chain fatty acids on the IC_{50}, Hill number, the concentrations that decreased (alcohols) or increased (fatty acids) phase transition temperature (ΔT) 1.0°C, and oil/water partition coefficients

	IC_{50} (μM)	n_H*	ΔT (μM)#	log P§
n-Alcohols				
(C10)	92.3	1.03	67.3	4.03
(C12)	22.0	1.07	27.1	5.08
(C14)	13.6	1.13	13.1	6.08
(C16)	22.8	1.09	21.0	7.08
(C18)	126.8	1.10	141.2	8.08
Fatty acids				
(C10)	13.2	0.83	7.4	0.30
(C12)	1.2	0.89	2.3	1.30
(C14)	0.68	0.89	1.2	2.31
(C16)	0.67	0.95	1.0	3.31
(C18)	0.63	0.95	0.87	4.31
Arachidonic acid	0.62	0.96	0.87	

*Hill Number.
#Ligand concentrations (μM) that changed the transition temperature by 1.0°C.
§Hansch and Clayton (1973).

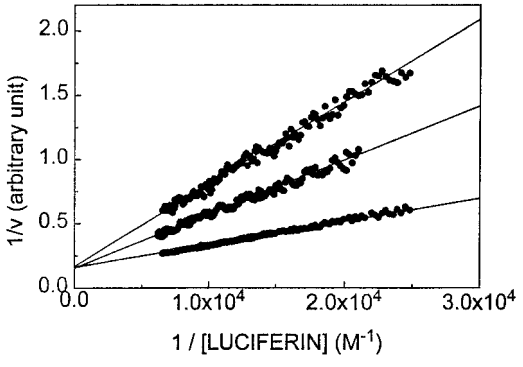

FIGURE 3 Lineweaver-Burk plots of myristic acid. Lineweaver-Burk plots were constructed by continuous infusion of luciferin under a steady-state condition by decelerating the reaction with the addition of pyrophosphate. The photomultiplier output was monitored by a Nicolet digital recording oscilloscope and downloaded onto floppy disks. The dilution effect (infusion volume) was corrected with a PC, and then the luciferin concentrations were converted into reciprocal form and extrapolated by linear regression with MicroCal Origin software. The lines are (*from the bottom*) control, 0.3 μM myristic acid, and 1.4 μM myristic acid. The K_i value was estimated by averaging three determinations. Myristic acid inhibited FFL in competition with luciferin with $K_i = 0.53$ μM.

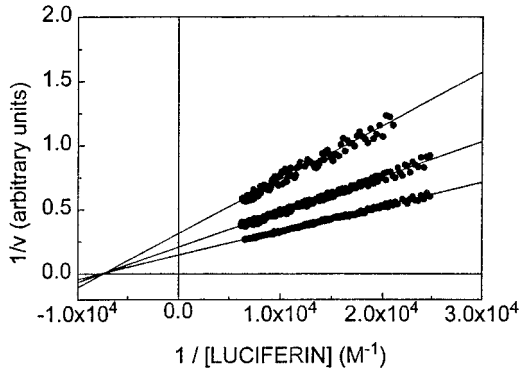

FIGURE 4 Lineweaver-Burk plots of myristyl alcohol. The lines are (*from the bottom*) control, 8.5 μM myristyl alcohol, and 21.1 μM myristyl alcohol. Myristyl alcohol inhibited FFL noncompetitively to luciferin with $K_i = 20.5$ μM.

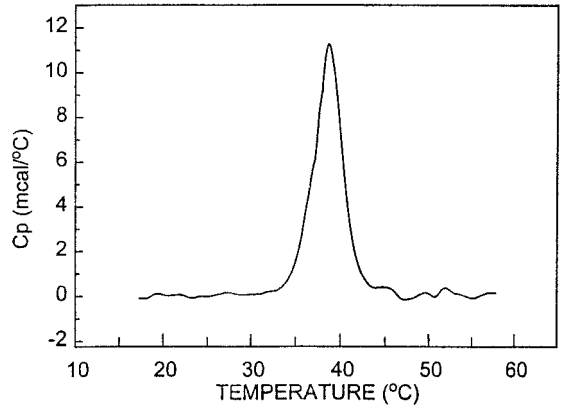

FIGURE 5 DSC thermogram of FFL. FFL was dissolved in 0.5 M glycylglycine buffer (pH 7.8) at 3 mg g^{-1} and scanned at 1.0°C min^{-1}.

lates the binding constant from the relation between the light intensity of the last stage and the luciferin concentrations at the first stage. In the multiple stages, there are ample sites that can interact with alcohols, and the validity of K_D is yet to be determined. The analysis for fatty acids, however, appears to be reliable because the action site is the initial stage (see below), where luciferin interacts with firefly luciferase.

Competition between fatty acids and luciferin may not be surprising, because the luciferin interaction with firefly luciferase involves activation of luciferin by ATP to form acylAMP between the carboxyl moiety of luciferin and the phosphate moiety of AMP. The sequence is identical to fatty acid activation by ATP. AcylCoA synthetases activate fatty acids to form acylAMP between the carboxyl moiety of fatty acids and the phosphate moiety of AMP. Firefly luciferase has wide homology with acylCoA synthetases (Suzuki et al., 1990; Babbitt et al., 1992; Ye et al., 1997). Presumably, the carboxyl moiety of fatty acids contacts the luciferin binding site. A lack of carboxyl moiety in alcohols makes these compounds incapable of finding precise luciferin recognition sites. With the above unity Hill coefficients, the action mode of alcohols is allosteric.

Differential scanning calorimetry (DSC) showed that thermal transition of firefly luciferase occurred at 38.7°C, with a transition ΔH_{cal} of 1.57 cal g^{-1} protein (Fig. 5). The cooling scan, however, did not show exothermic peaks. The transition was irreversible. Although the transition ΔH equals the heat absorbed during irreversible denaturation, the temperature profile is often distorted by the irreversible process. Kinetic theories have been proposed (Sánchez-Ruiz et al., 1988; Lepock et al., 1992; Hernándes-Arana, 1993; Shnyrov et al., 1996) to analyze the irreversible transition in large multidomain proteins according to the Lumry-Eyring (1954) rate process model, where reversible transition into multiple intermediate states is succeeded by the irreversible change, when the temperature is further increased.

The kinetic approach to irreversible transition measures the temperature dependence of the ratio between the folded native fraction and the irreversibly denatured fraction. Because of the irreversibility, the transition temperatures vary by the scanning rate and the protein concentration. The DSC data at 3.0 mg ml^{-1} protein are not directly comparable to those of a transmittance study at 0.27 mg ml^{-1} protein. Therefore, we compared the effects of protein concentration and scan rate on the transition temperature. At the transmittance scan rate of 1.0°C min^{-1}, the transition temperature was 41.5°C at 0.25 mg ml^{-1} and 38.6°C at 3.0 mg ml^{-1} protein. The transition temperature by DSC agrees with that of the transmittance scan. Although the transition temperature varies by the protein concentration and scan rate, the effects of ligands on the transition can be compared meaningfully at a fixed protein concentration and scan rate (Ueda and Suzuki, 1998). The effect on the transition temperature was compared by the temperature where the transition was half completed ($T_{1/2}$). Fig. 6 shows the dose-dependent effects of myristyl alcohol and myristic acid on the temperature scan of irreversible transition. The alcohols decreased, whereas fatty acids increased the $T_{1/2}$. The effects are expressed by the ligand concentrations that decreased the $T_{1/2}$ 1.0°C (Table 1). For example, myristyl alcohol decreased

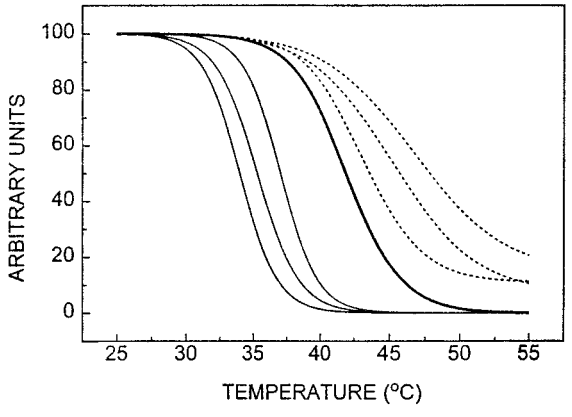

FIGURE 6 Temperature scan of the transmittance change of FFL. The lines are (*from the left*) 84 μM, 120 μM, and 150 μM myristyl alcohol, control (*thick line*), and 2.8 μM, 10 μM, and 21 μM myristic acid.

the $T_{1/2}$ at 13.1 μM, and myristic acid increased it at 1.2 μM. Arachidonic acid increased the $T_{1/2}$ at 0.87 μM.

ATP decreased the $T_{1/2}$ by 1.0°C at 5.01 mM, whereas luciferin increased it by 1.0°C at 0.6 mM. However, in the presence of 1.0 mM ATP, 2.3 μM luciferin was enough to increase the $T_{1/2}$ by 1.0°C. The mixture forms oxyluciferylAMP. The present result agrees with the report by DeLuca and Marsh (1967) that oxyluciferylAMP is a strong luciferin competitor with much higher affinity than luciferin, and protects the enzyme from irreversible thermal denaturation.

The dependence of the ligand effects on IC_{50} and transition temperature on the carbon-chain lengths is shown in Fig. 7. Potencies of the alcohols reached maximum at myristyl alcohol C(14). Further elongation of the carbon chain decreased the potency of the effect on both IC_{50} and $T_{1/2}$. The close similarity between the responses of the two parameters shows that the action mode is related to the unfolding of firefly luciferase. The carbon chain length, C(14), coincides with the cutoff point where further elongation of the alcohol carbon chain abolishes the anesthetic potency (Meyer and Hemmi, 1935; Alifimoff et al., 1989). In contrast, the potencies of fatty acids for both parameters increased monotonously with elongation of the carbon chain.

Koshland's transition-state theory (Koshland, 1958, 1963) postulates that binding of substrate induces the enzyme into a kinetically high-energy transition state to catalyze the reaction. Ligands that bind with high specificity stabilize the transition state (Chiou and Ueda, 1994; Catanzano et al., 1997). The increase in the thermal transition temperature by long-chain fatty acids indicates that the transition state is more stabilized than the native state to thermal change. High-affinity receptor binders protect the protein from thermal denaturation. They are structure makers.

A decrease in thermal transition temperature is equivalent to the freezing point depression, which is a colligative property and is nonspecific. The alcohols bind preferentially to the partially unfolded high-temperature state of the protein. They are structure breakers. Eyring (1966) proposed that anesthetics and alcohols unfold proteins by acting at the protein-water interface and change the structure of interfacial water cluster. The alcohols inhibit firefly luciferase allosterically by increasing the fraction of the partially denatured, less active state of firefly luciferase. The reversible unfolding destabilizes firefly luciferase to become vulnerable to the irreversible denaturation when the temperature is further elevated. Conversely, the long-chain fatty acids inhibited firefly luciferase by competing with luciferin and stabilized the transition state.

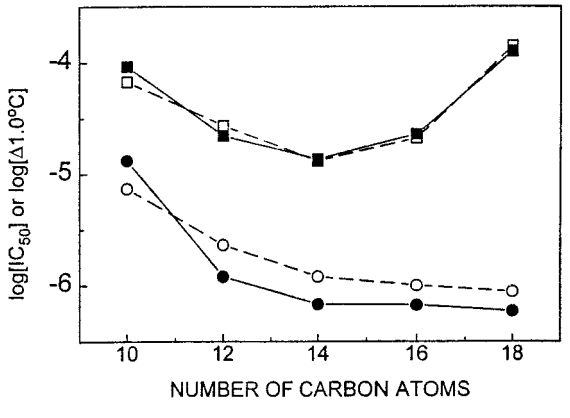

FIGURE 7 Dependence of IC_{50} and $T_{1/2}$ on the carbon chain lengths. Effects on the transition temperature are expressed by the ligand concentrations that decreased (*n*-alcohols) or increased (fatty acids) $T_{1/2}$ by 1.0°C. Squares represent *n*-alcohols and circles fatty acids. Closed symbols are IC_{50} and open symbols are effects on $T_{1/2}$.

The alcohols and anesthetics affect all macromolecules in water, whether they are proteins or lipid membranes, and inhibit all enzymes when their concentrations are increased. For example, the alcohols inhibit luminous bacteria and all other bioluminescent reactions. Fatty acids do not. In contrast, Ulitzur and Hastings (1978) showed that myristic acid stimulated the light intensity of aldehyde mutants of luminous bacteria. The actions of fatty acids on firefly luciferase are specific, whereas those of the alcohols and anesthetics are not.

Hydrophobic interactions are promoted by the van der Waals force, which is spatial. Therefore, the interaction is structure dependent, and each anesthetic or alcohol has its own affinity and preference for various proteins, even though the interaction is nonspecific. The reactions of a protein with these ligands vary by the resulting conformations and may not be identical. The important features of alcohols and anesthetics are the wide range of their actions on signal transduction systems and enzymes. For example, the papers presented at the New York Academy of Science Symposium on the effects of anesthetics and alcohols (Rubin et al., 1991) included $GABA_A$ and $GABA_B$ receptors, NMDA and non-NMDA receptors, glycine receptor, peptide receptor, G-protein-linked systems, α_2-adrenergic receptor, Na^+ channel, K^+ channel, Cl^- channel, protein kinase C, phospholipase C, inositide turnover, mitochondrial electron transport, transport ATPases, luciferases, etc., to name a few. The multitude of target systems is unique and exceptional. Receptor binders affect a single system; acetylcholine does not affect GABA receptors and GABA does not affect acetylcholine receptors, no matter how high the concentration is increased. We maintain that the actions of the anesthetics and alcohols are cooperative in multiple systems and nonspecific. The basic mechanism may be to dehydrate the macromolecular surface and destabilize protein structures by solvent effects. In this context, they are similar to the hydrophilic denaturants such as guanidine HCl and urea. These weak ligands must compete with water molecules because their affinities for proteins are not much higher than that of water, as described by the Wyman-Timasheff preferential binding model (Timasheff, 1992).

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