Does Pressure Antagonize Anesthesia? Opposite Effects on Specific and Nonspecific Inhibitors of Firefly Luciferase

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ABSTRACT Ueda and Suzuki (1998. *Biochim. Biophys. Acta.* 1380:313–319; 1998. *Biophys. J.* 75:1052–1057) reported that myristic acid inhibited firefly luciferase in μM range in competition with luciferin, whereas anesthetics inhibited it in millimeter ranges noncompetitively with luciferin. Myristate increased, whereas anesthetics decreased, the thermal denaturation temperature. The present study showed that high pressure increased the steady-state light intensity of the halothane-doped firefly luciferase but decreased that of the myristate-doped firefly luciferase. The steady-state light intensity showed a maximum at 19.1°C. At 19.1°C, high pressure did not affect the light intensity in the absence of the inhibitors. In the presence of 0.5 mM halothane, however, 25 MPa pressure (maximum effect) increased the light intensity to 106.0% of the control without the inhibitor. In the presence of 2.5 μM myristate, 40 MPa pressure decreased the light intensity to 90.9% of the control. When the temperature was 25°C in the absence of inhibitors, 40 MPa pressure increased the light intensity 119.2% of the ambient value. At 0.5 mM halothane, 40 MPa pressure further increased the light intensity to 106.1% above the control 40 MPa value. At 2.5 μM myristate, 40 MPa pressure decreased the light intensity to 90.1% of the control 40 MPa value. From the pressure dependence of the light intensity, the volume change ΔV of the enzyme was estimated at 25°C: 0.5 mM halothane increased $\Delta V = +3.93$ cm^3 mol^{-1}, whereas 2.5 μM myristate decreased $\Delta V = -7.66$ cm^3 mol^{-1}. Present results show that there are distinct differences between the specific and nonspecific ligands in their response to high pressure. Myristate, which competes with luciferin, decreased the protein volume and stabilized the conformation against thermal perturbation. Halothane, which does not compete with the substrate, increased the protein volume and destabilized the conformation.

INTRODUCTION

The first report that firefly luciferase was highly sensitive to anesthetics appeared more than three decades ago (Ueda, 1965). Subclinical concentrations of volatile anesthetics inhibited partially purified cell-free enzyme from Japanese firefly (*Luciola cruciata*). Later, Ueda and Kamaya (1973), reporting on the temperature dependence of the anesthetic effects on partially purified American firefly luciferase (*Photinus pyralis*), concluded that anesthetics inhibited the enzyme by reversibly denaturing the structure into the less active unfolded form. The ΔH of anesthetic interaction with the protein was about 370 kJ mol^{-1}. Dickinson et al. (1993) argued that the binding ΔH of anesthetics to firefly luciferase was smaller and negative, about -20 kJ mol^{-1}. However, their small negative value represents the transfer of anesthetic molecules ($\sim$ 100–200 Da) from water to firefly luciferase. Our large positive value represents the transfer of firefly luciferase (62,000 Da) from water to anesthetic solutions.

With differential scanning calorimetry, Chiou and Ueda (1994) reported that the thermal denaturation of firefly luciferase occurred at about 41°C with ΔH_{cal} of 415 kJ mol^{-1}. Two luciferin competitors, anilinonaphthalenesulfonate and toluidinonaphthalenesulfonate (DeLuca, 1969), increased the thermal denaturation temperature, whereas ethanol decreased it (Chiou and Ueda, 1994). Ueda and Suzuki (1998a,b) reported that myristate is three orders of magnitude stronger than volatile anesthetics and alcohols as an inhibitor of firefly luciferase. The Lineweaver-Burk plot at a steady-state condition showed that myristate inhibited firefly luciferase in competition with luciferin, whereas anesthetics did not. Luciferin is a heterocyclic carboxylate. The initial reaction of firefly luciferase is to activate luciferin by ATP to form acylAMP. Activation of fatty acids by acylCoA synthetases also involves activation of fatty acids by ATP to form acylAMP. Homology between these enzymes has been reported (Suzuki et al., 1990; Babbitt et al., 1992; Ye et al., 1997). It is not surprising that myristate competes with luciferin.

Moss et al. (1991) reported that application of high pressure did not affect the initial flash intensity of firefly luciferase. In contrast, Ueda et al. (1994) reported that high pressure decreased the steady-state light intensity at low temperatures but increased it at high temperatures. The discrepancy between Moss et al. (1991) and Ueda et al. (1994) was caused by the difference in the choice of parameters to represent the reaction velocity: initial flash intensity versus steady-state light intensity.

In ordinal enzyme kinetics, the rate is expressed by the amount of product accumulated in a unit of time. Because

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light intensity does not accumulate, the light intensity is equivalent to the differential of the ordinal enzyme reaction. The rapid reaction kinetics of the transition state theory (Hiromi, 1979) shows that when the rate of the first reaction in a multiple-stage reaction is faster than the rate of the final reaction, there is a rapid accumulation of the product of the first reaction. The rapid accumulation of the first product is designated a pre-steady-state burst. When the product accumulation is differentiated with regard to time, the initial rapid accumulation of the product is represented by a peak, equivalent to the initial flash intensity of firefly luciferase.

Firefly luciferase is a typical multiple-stage enzyme with three substrates, luciferin, ATP, and oxygen, interacting with the enzyme at different stages (McElroy and Seliger, 1962; DeLuca and McElroy, 1974, 1978).

$$E + ATP + Luciferin \rightleftarrows E \cdot LuciferylAMP + PPi$$

$$E \cdot LuciferylAMP + O_2 \rightarrow E \cdot OxyluciferylAMP + Light$$

$$E \cdot OxyluciferylAMP \rightarrow E + Oxyluciferin + AMP$$

$$E + Oxyluciferin + ATP \rightleftarrows E \cdot OxyluciferylAMP + PPi$$

where PPi is pyrophosphate. The first reaction is the production of photon, and the third reaction is the release of oxyluciferin from the enzyme. In the accompanying article (Ueda et al., 1999), the stopped-flow study showed that the initial peak intensity represents the concentration of the active enzyme and is unrelated to reaction kinetics. The present study compared effects of high pressure up to 40 MPa on the inhibitory effects of myristate and halothane. The steady-state condition was obtained by eliminating the initial peak with addition of pyrophosphate (McElroy and Seliger, 1962; Ueda et al., 1994; Ueda and Suzuki, 1998a,b).

MATERIALS AND METHOD

Lyophilized crystalline firefly luciferase from *P. pyralis*, D-luciferin, ATP, glycylglycine, and pyrophosphate were purchased from Sigma (St. Louis, MO), halothane from Imperial Chemical (New York, NY), and methanol from EM Science (Cherry Hill, NJ).

Steady-state light intensity was obtained by decelerating the initial reaction with addition of pyrophosphate. Firefly luciferase was dissolved in 100 mM glycylglycine buffer, pH 7.8, at 20 μg ml^{-1}. The firefly luciferase solution contained 100 μM luciferin, 10 mM $MgSO_4$, and 50 mM pyrophosphate, to which 5 mM ATP in the glycylglycine buffer was added.

The mixture was rapidly transferred to an Aminco high-pressure cell with sapphire windows (Silver Spring, MD). The cell was covered by a water jacket and the temperature was controlled by circulating water from a water bath. The cell temperature was monitored by a filament thermistor inserted into a small hole in the pressure block and a Digitech thermometer (United Systems, Dayton, OH) with 0.01°C resolution. The cell was pressurized with hexane via a separator. Pressure was monitored by an Autoclave Engineers model DPS-0201 transducer (Erie, PA) with 1.0 psi (0.07 bar) resolution.

The light intensity was measured by a Hitachi-Perkin-Elmer 139 UV-Visible spectrophotometer (Norwalk, CT). The sample compartment was replaced by the high-pressure cell. The photomultiplier output and the pressure-transducer output were recorded in a Nicolet 310 Digital Recording Oscilloscope (Madison, WI). The data were downloaded on floppy disks and analyzed by Origin software (Microcal, Northampton, MA).

RESULTS

The temperature scan of the steady-state light intensity of firefly luciferase showed that the maximum light intensity was observed at 19.1°C. When pressurized at the temperature that gave maximum light, hydrostatic pressure did not show any effect in the absence of inhibitors. The results agreed with our previous report (Ueda et al., 1994). When halothane was added, pressure increased the light intensity with a maximum effect at about 25 MPa (Fig. 1). Further increase of pressure decreased the light intensity to the control. At the halothane concentrations of 0.01, 0.05, 0.1, 0.25, and 0.5 mM, 25 MPa pressure increased the steady-state light intensity to 101.1, 102.2, 103.2, 104.5, and 106.0% of the control, respectively.

Fig. 2 shows the pressure effect on the steady-state light intensity in the presence of myristate at 19.1°C. Application of hydrostatic pressure in the presence of myristate decreased the light intensity monotonously and no minimum was observed, in contrast to the observed effect of halothane. At the myristate concentrations of 0.01, 0.25, 0.5, 1.0, and 2.5 μM, 40 MPa pressure decreased the steady-state light intensity to 98.9, 96.9, 95.1, 93.2, and 90.9% of the control, respectively.

Fig. 3 shows the pressure effect on the steady-state light intensity at 25°C. At this temperature, 40 MPa pressure increased the steady-state light intensity 119.2% of the control at ambient pressure (0.1 MPa) in the absence of the inhibitors. Application of hydrostatic pressure in the presence of halothane further increased the steady-state light intensity monotonously without a maximum. At halothane concentrations of 0.01, 0.05, 0.1, 0.25, and 0.5 mM, 40 MPa pressure increased the steady-state light intensity, respectively, to 101.4, 102.5, 103.6, 104.9, and 106.1% of the control without the anesthetic at 40 MPa. Fig. 4 shows the pressure effect on the myristate inhibition at 25°C. At the myristate concentrations of 0.05, 0.25, 0.5, 1.0, and 2.5 μM, 40 MPa pressure decreased the steady-state light intensity to 98.6, 96.5, 94.4, 92.4, and 90.1% of the control, respectively.

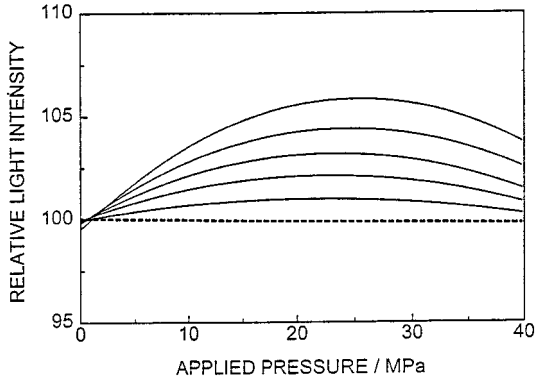

FIGURE 1 Effect of hydrostatic pressure at 19.1°C on the steady-state light intensity in the presence of halothane. Ordinate is the relative light intensity, taking the values at ambient pressure 100. Abscissa is the applied hydrostatic pressure in MPa. The lines are (from the bottom): control, halothane 0.01, 0.05, 0.1, 0.25, and 0.5 mM. The pressure effect on halothane showed a maximum at about 19°C.

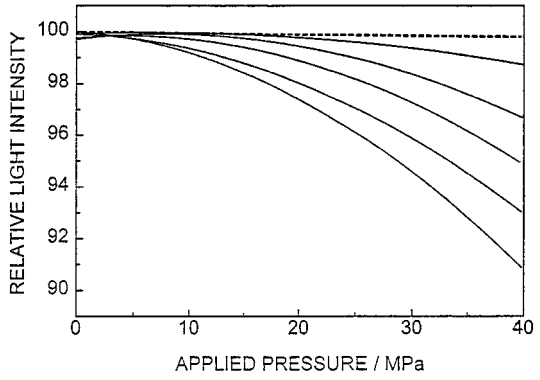

FIGURE 2 Effect of hydrostatic pressure at 19.1°C on the steady-state light intensity in the presence of myristate The lines are (from the top): control, myristate 0.01, 0.25, 0.5, 1.0, and 2.5 μM. The pressure effect on myristate inhibition was monotonous, without minimum or maximum, and decreased the light intensity.

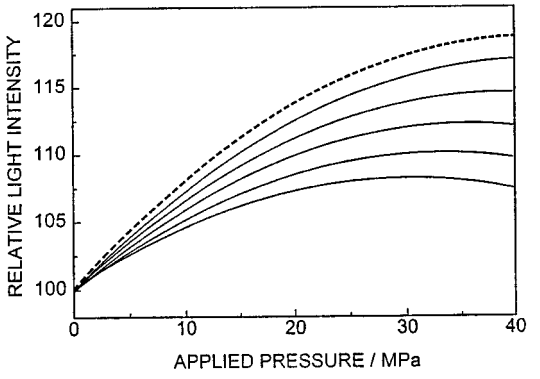

FIGURE 4 Effect of hydrostatic pressure at 25°C on the steady-state light intensity in the presence of myristate The lines are (from the top): control without inhibitor, myristate 0.01, 0.25, 0.5, 1.0, and 2.5 μM. The pressure effect on myristate inhibition was monotonous, without minimum or maximum, and decreased the light intensity.

There are two reference states (controls) to express the pressure effects on the light intensity of the ligand-bound firefly luciferase: first, the light intensity at the same pressure in the absence of inhibitors; second, the light intensity at ambient pressure in the presence of inhibitors. The accompanying article expressed the pressure effect according to the second criterion. When compared with halothane-inhibited steady-state light intensity at ambient pressure, 40 MPa pressure increased the steady-state light intensity 120.9, 122.1, 123.5, 125.0, and 126.4% of the control, respectively. Similarly, when compared with the myristate-inhibited light intensity at ambient pressure, 40 MPa pressure increased the light intensity to 117.1, 114.6, 112.1, 109.7, and 107.5% of the control, respectively. These increments are smaller than the control.

From the pressure effects on the light intensities of the control and the inhibitor-bound luciferase, the volume changes of the host protein were estimated. The Johnson-Eyring rate-process model calculates the ΔV by plotting the logarithm of the ratio between the inhibitor-induced decrement of the light intensity and the control light intensity against the applied pressure (Appendix). Fig. 5 shows the Johnson-Eyring plot of 0.5 mM halothane at 25°C. From this slope, the ΔV was estimated to be $+3.94$ cm^3 mol^{-1}. Fig. 6 is the plot of 2.5 μM myristate at 25°C. The ΔV was -7.66 cm^3 mol^{-1}.

DISCUSSION

Application of high pressure clearly distinguished the competitive inhibitors from the noncompetitive inhibitors. Pressure antagonized the effect of the nonspecific inhibitor, whereas it enhanced the effect of the competitive inhibitor. At the temperature that gave the maximum light intensity, hydrostatic pressure did not affect the light intensity. Even at this temperature, high pressure antagonized the effect of the noncompetitive inhibitor, halothane, on the steady-state luminescence, whereas it intensified that of the competitive inhibitor, myristate.

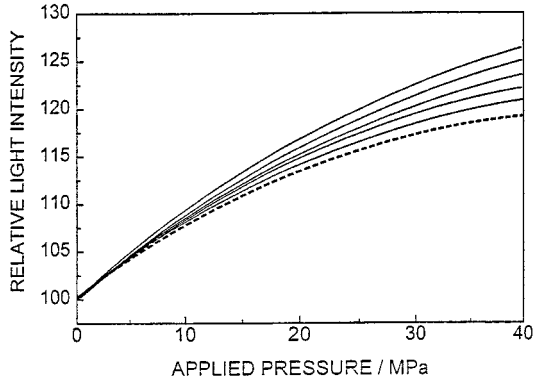

FIGURE 3 Effect of hydrostatic pressure at 25°C on the steady-state light intensity in the presence of halothane. The lines are (from the bottom): control without inhibitor, halothane 0.01, 0.05, 0.1, 0.25, and 0.5 mM. The pressure effect on halothane became monotonous and increased the light intensity.

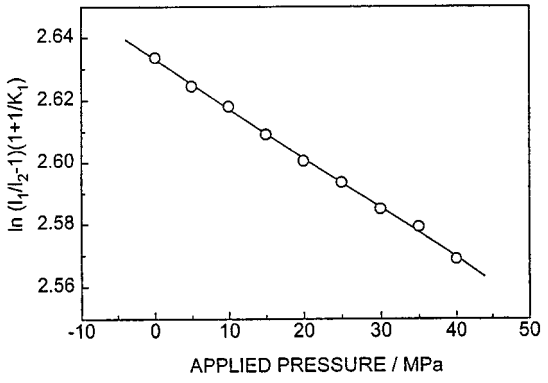

FIGURE 5 The Johnson-Eyring plot at 25°C for 0.5 mM halothane. Ordinate is the logarithm of $(I_1/I_2 - 1)(1 + 1/K_1)$, where I_1 is the steady-state light intensity of the control, I_2 is the light intensity in the presence of halothane, and K_1 is the equilibrium constant between the native and reversibly unfolded states of firefly luciferase (Appendix). Abscissa is the applied hydrostatic pressure in MPa.

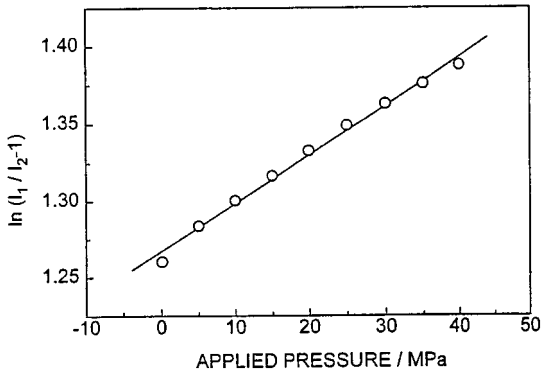

FIGURE 6 The Johnson-Eyring plot at 25°C for 2.5 μM myristate. Ordinate is the logarithm of $(I_1/I_2 - 1)$, where I_1 is the steady-state light intensity of the control, I_2 is the light intensity in the presence of myristate. Because the activity of the competitive inhibitors does not depend on unfolding, the $(1 + 1/K_1)$ term is not included (see Appendix).

Eyring (1966) proposed the rate-process theory according to which nonspecific binders (anesthetics and alcohols) interact with enzymes by nonspecific hydrophobic effect and reversibly unfold the enzyme. Koshland (1958, 1963) proposed the induced-fit theory, according to which the specific binding of substrates to enzymes induces the enzyme conformation into the high-energy transition state. We also found that the effects of specific and nonspecific binders on the physical properties of the host protein are reverse. Chiou and Ueda (1994) and Ueda and Suzuki (1998a,b) showed that the luciferin competitors increased the thermal denaturation temperature of firefly luciferase. Catanzano et al. (1997) also showed that D-glucose increased the denaturation temperature of yeast hexokinase B. DeLuca and Marsh (1967) showed that oxyluciferylAMP is a strong luciferin competitor and protected firefly luciferase from thermal inactivation. In contrast, the nonspecific inhibitors of firefly luciferase, anesthetics and alcohols, decreased the thermal denaturation temperature (Chiou and Ueda, 1994; Ueda and Suzuki, 1998a,b). Velicelebi and Sturtevant (1979) showed that alcohols decreased the thermal denaturation temperature of lysozyme.

TABLE 1 The effect of hydrostatic pressure on the steady-state light intensity of firefly luciferase, inhibited by halothane or myristate, at 19.1 and 25.0°C

	Halothane (mM)				
	0.01	0.05	0.10	0.25	0.50
19.1°C*	101.1	102.2	103.2	104.5	106.0
25.0°C†	101.4	102.5	103.6	104.9	106.1
	Myristate (μM)				
	0.10	0.25	0.5	1.0	2.5
19.1°C*	98.9	96.9	95.1	93.2	90.9
25.0°C†	98.6	96.5	94.4	92.4	90.1

The values are expressed as a percent of the control at the same pressure without the ligands.
*Pressurized at 25 MPa, where the pressure effects were maximum.
†Pressurized at 40 MPa.

The present study showed that myristate decreased the volume of firefly luciferase, whereas halothane increased the volume. By solution densimetry, Ueda and Mashimo (1982) showed that anesthetics expanded the partial molal volume of bovine serum albumin. The transition state, proposed by Koshland (1958, 1963), resists thermal denaturation and has compact conformation compared to the native state. The unfolded state has expanded conformation and lower thermal denaturation temperature. These results demonstrate that anesthetics and alcohols are nonspecific conformation destabilizers, whereas specific binders are conformation stabilizers.

To our knowledge, all reports but one on the anesthetic actions on enzymes concluded they are allosteric effects. The only exception, concluding that anesthetics compete with substrates (Franks and Lieb, 1984), was based on use of the Lineweaver-Burk plots on the pre-steady-state flash intensity and is therefore incorrect. Aside from the use of the nonsteady state in analyzing the inhibition kinetics, the use of the Lineweaver-Burk plot to determine multiple-stage reactions requires justification for the applicability of the linearization procedure. The reaction rate law of enzyme action is highly complicated, as described by the classical theory of King and Altman (1956) and by the recent theory of Roussel and Fraser (1993). In addition to the complexity of the rate law, there are a number of intermediate states between the luciferin binding and the light emission: binding of ATP, binding of O_2, oxidation of luciferin, deprotonation, transition to the excited state, energy release by returning to the ground state, and release of the final product, oxyluciferin, from the enzyme. Anesthetics and alcohols may act at any one of these stages. Even if the plot shows apparent competition, the exact binding mode may not be established. The Lineweaver-Burk plot determines only whether or not the inhibition is surmountable by the infinite increase of the substrate concentration. Its indiscriminate use leads to erroneous conclusions.

At present, the idea that anesthetics and alcohols bind to a specific receptor is widely accepted without clear definition of specificity and nonspecificity. Specific binding means that a specific compound interacts with a specific receptor on a specific protein. Acetylcholine does not bind to γ-aminobutyric acid receptors no matter how much the concentration is increased. Also, γ-aminobutyric acid does not bind to acetylcholine receptors. The neurotransmitters are specific binders. Anesthetics affect γ-aminobutyric acid receptors, acetylcholine receptors, and all other signal transduction systems and channels when the concentration is increased. They are nonspecific binders; therefore, anesthetics inhibit bacterial luciferase as well. Myristate is a specific inhibitor. Therefore, it does not inhibit bacterial luciferase. Instead, myristate activated the dark mutant of bacterial luciferase (Ulitzur and Hastings, 1978).

As a rule, specific binders are characterized by high affinity and small binding numbers, whereas nonspecific

binders are characterized by low affinity and large binding numbers. Anesthetics fit well within the concept of nonspecific ligands. For example, Yoshida et al. (1997) reported that the weak binding of halothane to bovine serum albumin was associated with K_D 10.9 mM and the maximum binding number, B_{max}, of 34.5 by ^{19}F-NMR. The effects of specific ligands on firefly luciferase temperature and pressure are unambiguously the reverse of the effects of nonspecific ligands.

The strong hydrophobicity of the luciferin binding site (DeLuca, 1969) makes it possible to find anesthetic molecules at the site. Nevertheless, they do not interact with the luciferin receptor. There is a major difference between residing at the receptor site and interacting with the receptor. The surface of a protein is composed of hydrophobic and hydrophilic sites. These sites must be occupied by either solvent or cosolvent. Anesthetics are nonspecific hydrophobic binders and interact with all accessible hydrophobic sites on proteins. Ueda and Suzuki (1998a,b) have shown that the effects of anesthetics are highly cooperative with Hill numbers above unity. The Hill number does not indicate the actual number of ligands that bind to the host molecule. The derivation assumes highly cooperative interaction between enzyme (E) and ligand (L) where only two species are present, E and EL_n. The intermediates, EL_1, EL_2, $\cdots$, EL_{n-1}, are not counted. When the Hill number exceeds one, at least two ligand molecules bind to the host molecule. Therefore, the Hill number is often called the cooperativity parameter. We maintain that anesthetics act by cooperative actions at multiple sites, rather than interaction with a single specific site.

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Appendix

We (Ueda and Suzuki, 1998a,b) reported that myristic acid inhibited firefly luciferase by competing with the substrate luciferin. Conversely, anesthetics inhibited firefly luciferase noncompetitive with luciferin, and by changing the equilibrium constant, K_1, between the unfolded inactive fraction and native fraction.

We designate X and r as the concentration and the number, respectively, of bound competitive inhibitors. U and s are, respectively, the concentration and the number of bound allosteric inhibitors. K_2 and K_3 are the association constants of the competitive and noncompetitive inhibitors, respectively. I_1 and I_2 are the light intensity of the control and the inhibitor-bound firefly luciferase, respectively.

Because myristate inhibits firefly luciferase by competing with luciferin and not by thermal inactivation, the ratio of the light intensities of firefly luciferase in the absence (I_1) and presence (I_2) of myristate is expressed according to the Johnson-Eyring rate-process theory (Ueda et al., 1994):

$$\frac{I_1}{I_2} = 1 + K_2(X)^r$$

$$\ln\left(\frac{I_1}{I_2} - 1\right) = r \ln(X) + \ln K_2 = r \ln(X) - \frac{\Delta G_{0,2}}{RT} - \frac{p\Delta V_2}{RT}$$

Anesthetics inhibit the enzyme by changing the equilibrium constant, K_1, of the unfolded inactive fraction and native fraction.

$$\ln\left(\frac{I_1}{I_2} - 1\right)\left(1 + \frac{1}{K_1}\right) = s \ln U + \ln k_3$$

$$= s \ln U - \frac{\Delta G_{0,3}}{RT} - \frac{p\Delta V_3}{RT}$$

where subscript 0 signifies zero applied pressure and subscripts 2 and 3 are the properties related to myristate and anesthetics, respectively.

The ΔV values are estimated by plotting the left-hand side against the applied pressure.

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