Does Pressure Antagonize Anesthesia? High-Pressure Stopped-Flow Study of Firefly Luciferase and Anatomy of Initial Flash

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ABSTRACT The antagonizing effect of high pressure against anesthesia is well known. With purified firefly luciferase, however, Moss et al. (1991. *Biophys. J.* 60:1309–1314) reported that high pressure did not affect the initial flash intensity. Firefly luciferase emits a burst of light when the substrates luciferin and ATP are added in the presence of O_2. The light intensity decays rapidly and the weak light lasts for hours. The initial flash is a transient event and is not in a steady state. The steady state is represented by the slope of the linear part of the integral of the light output. The present study used a high-pressure stopped-flow system to compare the pressure effects on the initial flash intensity and the steady-state light intensity. The flash intensity did not change by the application of hydrostatic pressure in the presence or absence of chloroform or 1-octanol. In contrast, high pressure increased the steady-state light intensity. The application of 12 MPa pressure increased the steady-state light intensity of firefly luciferase inhibited by 5 mM chloroform or 0.7 mM 1-octanol by 19.7% and 18.8%, respectively. When analyzed by the rapid reaction kinetics of the transition state theory, the initial peak intensity represents the total amount of active enzyme and is unrelated to the reaction rate. Anesthetics inhibited the initial flash by unfolding the protein, thereby decreasing the concentration of the active enzyme. Pressure affected the steady-state light intensity by changing the reaction rates.

INTRODUCTION

The antagonizing effect of high pressure on anesthesia was first reported in the light intensity of luminous bacteria (Johnson et al., 1942a,b; Eyring and Magee, 1942; Brown et al., 1942). Johnson and Flagler (1951) responded to criticism that the light intensity of bacteria is irrelevant to the anesthesia mechanism by showing that tadpoles anesthetized with ethanol or urethane started swimming again when hydrostatic pressure of 10 MPa was applied. The pressure reversal of anesthesia has been generally accepted as a standard feature of anesthetic actions. With purified firefly luciferase, however, Moss et al. (1991) reported that high pressure did not affect the initial flash intensity in the presence or absence of anesthetics.

When ATP is added to firefly luciferase in the presence of luciferin and oxygen, a flash of light is observed after 25 ms of complete darkness and reaches a maximum intensity at about 300 ms (DeLuca and McElroy, 1974). The light intensity rapidly decays to a low level and stays there for several hours. Because the light intensity changes rapidly with time, the meaning of the peak is unknown and the initial peak intensity does not represent the steady-state reaction rate. Because all equations of enzyme kinetics are constructed on the steady-state condition, it is necessary to estimate the steady-state reaction rate. There are several methods of estimating the steady-state reaction rate of firefly luciferase. These include scintillation counting, calculating the slope of the straight part of the integral of light output, and deceleration of the reaction rate by pyrophosphate to obtain steady-state light intensity.

The rapid-reaction kinetics of the transition state theory (Hiromi, 1979) shows that when the rate of the initial reaction to form the first product (P_1) is faster than that of the final reaction to form the last product (P_2) (dissociation of the final product from the enzyme) in a multiple-stage system, P_1 accumulates rapidly at the beginning (see Appendix). When the product accumulation is differentiated with respect to time, the initial steep accumulation is represented by a peak, which is designated as pre-steady-state burst. Firefly luciferase is a typical multistage enzyme, P_1 is the photon production, and P_2 is the release of the final product, oxyluciferin, from the enzyme. In a great excess of the substrate concentration over the enzyme concentration, $[S] \gg [E]_0$, the steady-state reaction rate is expressed by the slope of the straight part of the integral of the light output (Appendix).

Inhibitor kinetics must be analyzed under steady-state conditions. To evaluate whether the initial peak represents reaction rates, the present study used a custom-built high-pressure stopped-flow system to analyze the rapid change of luminescence. It will be shown that the initial flash intensity does not respond to high pressure, but the steady-state light intensity does.

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EXPERIMENTAL PROCEDURE

Lyophilized crystalline firefly luciferase from *Photinus pyralis*, Na_2ATP, D-luciferin, and glycylglycine were obtained from Sigma (St. Louis, MO). Chloroform (99.8% minimum, stabilized with ethanol) and 1-octanol were obtained from Nakarai Chemical (Kyoto, Japan).

Rapid reaction kinetics of chloroform and 1-octanol on the light intensity of firefly luciferase was measured by two stopped-flow spectrophotometers. The ambient pressure kinetics was measured by a pneumatically driven stopped-flow spectrophotometer (Otsuka Denshi Model RA-401, Osaka, Japan). The mixing is 99.5% complete within 2 ms.

The high-pressure stopped-flow apparatus was custom-built by Hikari High-Pressure Technologies (Hiroshima, Japan) according to the design of Ishihara et al. (1982). It consists of two pressure-resistant injection syringes, a thermostated optical cell with a pressure sensor and a mixer, and a pneumatically driven Silicone KF 96-filled (Shin-Etsu Chemical, Tokyo, Japan) pressure system with pressure intensifier with an area ratio of 200. The high-pressure optical cell has a light path length of 1.0 cm and is made of Diflon (polychlorotrifluoroethylene, Daikin Co., Osaka, Japan) with sapphire windows. The mixing is 99.5% complete within 3 ms.

Firefly luciferase was dissolved at 10 μg ml^{-1} in a 25-mM glycylglycine buffer, pH 7.8. The ATP-luciferin mixture contained 4 mM Na_2ATP, 14 mM $MgSO_4$, and 82.5 μM luciferin in the same buffer. Chloroform and 1-octanol were added to the ATP-luciferin solution by a microsyringe. Both solutions were mixed at a ratio of 1:1 by volume. Final concentrations were 41.25 μM luciferin, 2 mM ATP, 7 mM $MgSO_4$, and 5 μg/ml^{-1} buffer firefly luciferase. The sample temperature, controlled by circulating water around the system from a water bath of constant temperature, was maintained at 25 $\pm$ 0.1°C. Pressure effects were measured at 2, 5, 8, 12, and 15 MPa. The photomultiplier output was recorded in a Nicolet 310 digital oscilloscope (Nicolet, Madison, WI).

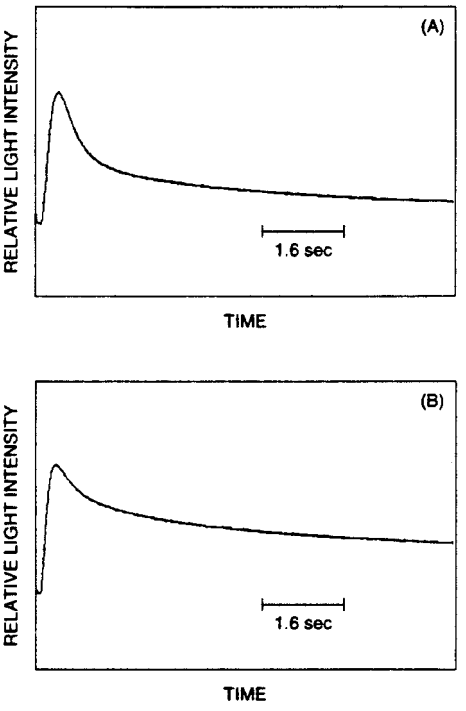

FIGURE 1 Stopped-flow tracings of the effect of 1-octanol on the light intensity of firefly luciferase at ambient pressure and 25°C. (*A*) control. (*B*) 0.7 mM 1-octanol.

RESULTS

Fig. 1 shows the stopped-flow tracings of the light intensity at 25°C in the absence (*A*) and presence (*B*) of 0.7 mM 1-octanol at ambient pressure. Fig. 2 shows the same at 12 MPa (Fig. 2, *A* and *B*). With or without applied pressure, the initial peak intensities in the presence of 0.7 mM 1-octanol were 36.8% of the control and in the presence of 5 mM chloroform, 10.7% of the control. The peak intensity data of 5 mM chloroform and 0.7 mM 1-octanol at 25°C and at hydrostatic pressures of 2, 5, 8, 12, and 15 MPa are compiled in Table 1. The enzyme activity is expressed by the ratio to the control without anesthetics. One-way analysis of variance showed that the differences among pressures were not significant: $p = 0.081$ for chloroform and $p = 0.083$ for 1-octanol. Fig. 3 illustrates the pressure effects, with filled circles representing the effects of chloroform and open circles the effects of 1-octanol. Hydrostatic pressure up to 15 MPa did not affect the peak intensity of the anesthetic-inhibited firefly luciferase.

Fig. 4 is a combined figure of tracings of the effects of 0.7 mM 1-octanol at ambient pressure and at 12 MPa, replotted from Figs. 1 *B* and 2 *B*, respectively. Although the initial peak heights are similar, the tailings are different. The stopped-flow tracings were integrated to analyze the steady-state reaction rates according to the rapid reaction kinetics.

Fig. 5 is the integral of Fig. 4. The slope of the linear part of the integrated light intensity was 37.6 at ambient pressure and 44.5 at 12 MPa. The slope of the integrated light output at 12 MPa was 18.2% steeper than that at ambient pressure. In 5 mM chloroform solution, the slope at the ambient pressure was 71.1 and the slope at 12 MPa was 85.1. Hydrostatic pressure of 12 MPa increased the light intensity about 19.7%.

DISCUSSION

The present result demonstrates the importance of the steady-state condition to analysis of reaction kinetics. High pressure in the range of $\sim$ 10–15 MPa did reverse anesthesia when analyzed by the steady-state light intensity. Johnson et al. (1942a,b) recognized pressure reversal in bacterial luciferase because they used the steady-state light intensity, whereas Moss et al. (1991) did not recognize it in firefly luciferase because they used the non-steady-state light intensity.

Although pressure did not affect initial peak intensity, anesthetics decreased the initial peak (Ueda, 1965; Franks and Lieb, 1984). The precise meaning of the peak intensity is unknown. When the light intensity is integrated with respect to time, the slope of the linear part represents the steady-state reaction rate. The intercept of the extrapolation of the linear line at the *y*-axis is designated π (Fig. 5). The initial flash intensity closely represents the π value.

Eq. 10 in the Appendix describes the structure of π.

$$\pi = \left(\frac{k_{+2}}{k_{+2} + k_{+3}}\right)^2 \left(\frac{s_0}{K_m + s_0}\right)^2 e_0$$

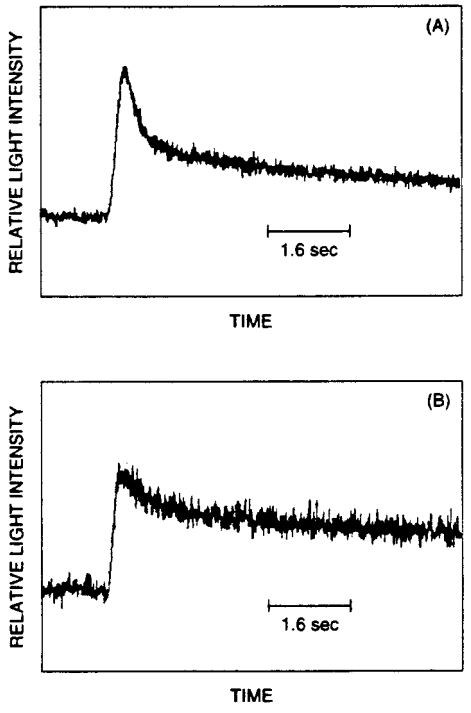

FIGURE 2 Stopped-flow tracings of the effect of 1-octanol on the light intensity of firefly luciferase at 12 MPa pressure and at 25°C. (*A*) control. (*B*) 0.7 mM 1-octanol. The S/N ratio of the high-pressure stopped-flow system is inferior to the ambient stopped-flow system shown in Fig. 1.

where k_{+2} is the rate of photon production, k_{+3} is the rate of the oxyluciferin releasing reaction, s_0 is the total substrate concentration, and e_0 is the total active enzyme concentration.

$$e_0 = e + [ES] + [ES']$$

The equation shows that $\pi = e_0$ when $k_{+2} \gg k_{+3}$ and $s_0 \gg K_m$. This means that the amount of the total active enzyme, e_0, did not change appreciably by the applied pressure of moderate ranges under the present experimental condition. Pressure affects the steady-state light intensity by $k_{+2}[ES]$ according to Eq. 15. Anesthetics inhibited the initial flash intensity by decreasing the amount of the active enzyme by reversibly unfolding the enzyme into the less active state. The initial flash intensity is unrelated to the reaction rate. The flash intensity reports the state of the enzyme, not the reaction rate. The rapid reaction kinetics of the transition rate theory treats enzymes as a reactant, whereas ordinary kinetic theories treat enzymes as catalysts.

TABLE 1 The effects of high pressure up to 15 MPa on the initial flash intensity

Pressure (MPa)	2	5	8	12	15
1-Octanol	0.374	0.341	0.352	0.368	0.376
SD	0.069	0.022	0.022	0.024	0.087
Chloroform	0.115	0.117	0.089	0.107	0.108
SD	0.022	0.026	0.006	0.013	0.021

The flash intensity is expressed by the ratio to the control without the inhibitors at the same pressure. 1-Octanol concentration was 0.7 mM; chloroform concentration was 5 mM. Temperature, 25°C.

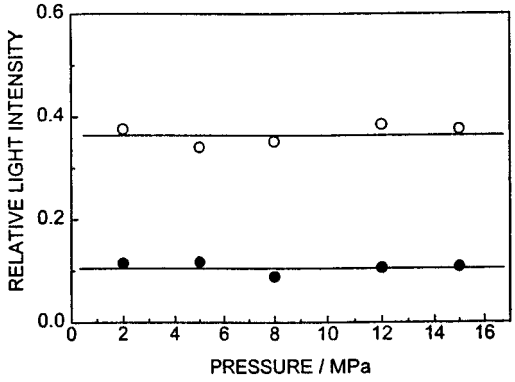

FIGURE 3 Response of the initial peak luminescence intensity under anesthesia at 25°C, at hydrostatic pressure 2 to 15 MPa. Open circles are for 0.7 mM 1-octanol solution and closed circles are for 5 mM chloroform solution. The differences among pressures are obviously not significant. One-way analysis of variance showed $p = 0.081$ for 1-octanol and $p = 0.083$ for chloroform.

There are several reports showing that pressure did not antagonize anesthesia in freshwater shrimp, *Gammarus pulex* (Smith et al., 1984, 1986) and in *Caenorhabditis elegans* (Eckenhoff and Yang, 1994). These negative reports indicate that the pressure effects on living creatures are not due to the pain and discomfort caused by high pressure and that the biphasic effect of pressure, separated by the temperature at the maximum activity (Ueda et al., 1994) where high pressure decreases the biological activities at the temperatures below that gives the maximum activity, may also be operative in the activity of living creatures. It is noteworthy that these negative reports were obtained at the conditions where pressure decreased the animals' activity in the absence of anesthetics. Simon et al. (1983) reported that pressure antagonized anesthesia in brine shrimp, *Artemia salina*, despite high pressure decreased EC_{50} (the concentration that anesthetizes 50% of the population) of the anesthetic. This was concluded on the

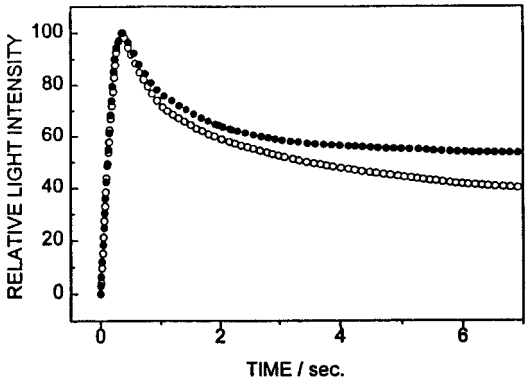

FIGURE 4 Combined figure of the effect of 1-octanol re-plotted from Figs. 1 and 2. *Open circles*: ambient pressure. *Closed circles*: 12 MPa.

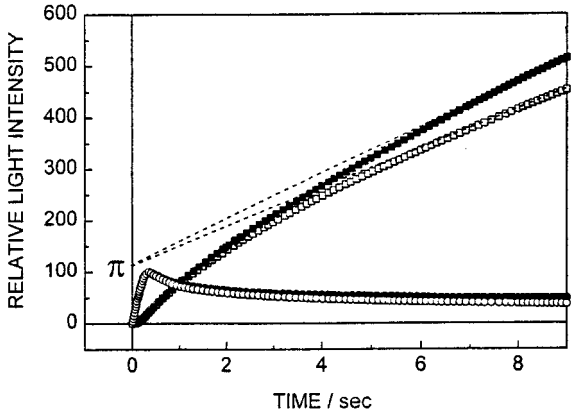

FIGURE 5 Integral of the stopped-flow tracings of Fig. 4. Open symbols are ambient pressure and closed symbols are at 12 MPa. Squares are the integration of Fig. 4 and circles represent Fig. 4. The slope of the linear parts of the integral of 12 MPa and ambient pressure data were 56.2 and 47.3, respectively. The extrapolation of the slope of the linear part intercepted the y axis (designated as π) at about 108.

ground that the magnitude of the decrease of EC_{50} at high pressure was less than the arithmetic sum of the pressure-induced and anesthetic-induced decreases of the activity. A similar conclusion was reached with respect to the pressure effect on bacterial luminescence (Nosaka et al., 1988). The pressure reverses anesthesia when the plot between the activity (enzyme or animal movement) ratio (activity under anesthesia)/(activity of the control) and applied pressure shows a positive slope, i.e.,

$$\left(\frac{\partial(I_A/I_0)}{\partial P}\right)_T > 0$$

where I_A is the activity in the presence of anesthetics and I_0 is the control activity without anesthetics.

The pressure antagonism of anesthesia appears to have no exceptions. Together with the fact that anesthetics affect almost all proteins and lipid membranes, it is probably safe to conclude that the anesthetic action is physical and nonspecific.

APPENDIX

The three-stage reaction is written,

$$E + S \underset{k_{-1}}{\overset{k_{+1}}{\rightleftarrows}} ES \xrightarrow{k_{+2}} ES' + P_1 \qquad ES' \xrightarrow{k_{+3}} E + P_2 \quad (1)$$

In the firefly luciferase reaction, P_1 is the emitted photon, and P_2 is the oxyluciferin released from firefly luciferase.

When the substrate concentration greatly exceeds the enzyme concentration, $[S] \gg [E]_0$, and with respect to the early part of the reaction, $s = s_0$, the following equations are obtained (Hiromi, 1979):

$$[ES] \equiv x = (k_{+3}Ae_0/\lambda)[1 + A(k_{+2}/k_{+3})e^{\lambda t}] \quad (2)$$

$$[ES'] \equiv y = (k_{+2}Ae_0/\lambda)(1 - e^{-\lambda t}) \quad (3)$$

where s_0 is the total substrate concentration, e_0 is the total enzyme concentration $e_0 = e + x + y$, and A and λ are, respectively,

$$A = \frac{s_0}{K_s' + s_0} \quad \text{where } K_s' = \frac{k_{-1} + k_{+2}}{k_{+1}} \quad (4)$$

and

$$\lambda = \frac{k_{+3} + k_{+2}s_0}{K_s' + s_0} \quad (5)$$

Because the present study concerns the steady-state condition, the exponential term reduces to approximately zero, and $[ES] = x$ becomes constant. By substituting A and λ,

$$x \approx \frac{k_{+3}e_0s_0}{k_{+3}(K_s' + s_0) + k_{+2}s_0} \quad (6)$$

The time-dependent change in the products, P_1 and P_2 are, respectively,

$$[P_1] \equiv p_1 = v_0t + \pi(1 - e^{-\lambda t}) \quad (7)$$

and

$$[P_2] \equiv p_2 = v_0t - (v_0/\lambda)(1 - e^{-\lambda t}) \quad (8)$$

where v_0 and π are, respectively,

$$v_0 = k_0e_0s_0/(K_m + s_0) \quad (9)$$

and

$$\pi = \left(\frac{k_{+2}}{k_{+2} + k_{+3}}\right)^2\left(\frac{s_0}{K_m + s_0}\right)^2 e_0 \quad (10)$$

The v_0 is the rate when the reaction has reached the steady-state condition. The relation between $[P_1]$ and π is shown in Fig. 5. The K_m and k_0 at the steady-state condition are, respectively,

$$K_m = \left(\frac{k_{-1} + k_{+2}}{k_{+1}}\right)\left(\frac{k_{+3}}{k_{+2} + k_{+3}}\right) = K_s'\left(\frac{k_{+3}}{k_{+2} + k_{+3}}\right)$$

$$k_0 = \frac{k_{+2}k_{+3}}{k_{+2}+k_{+3}} \quad (11)$$

As before, we focused on the steady state rather than the pre-steady state. The exponential term becomes approximately zero, and both P_1 and P_2 become linear functions of the time. Here, we consider only the slope, v_0. From Eqs. 9 and 11,

$$v_0 = \frac{(k_{+2} + k_{+3})k_0e_0s_0}{k_{+3}(K_s' + s_0) + k_{+2}s_0} \quad (12)$$

Comparing Eq. 6 and Eq. 12, the relation between x and v_0 becomes

$$v_0 = k_{+2}x \quad (13)$$

Similarly, the relation between y and v_0 is

$$v_0 = k_{+3}y \quad (14)$$

These results on x and y are equivalent to the values after the steady-state approximation of [ES] and [ES′]:

$$v \equiv d[P_1]/dt = d[P_2]/dt = k_{+2}[ES] = k_{+3}[ES'] \quad (15)$$

where v is the velocity when the reaction has reached the steady-state condition.

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