Temperature-Dependent Effects of High Pressure on the Bioluminescence of Firefly Luciferase

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ABSTRACT This study measured the effect of high pressure on the enzyme kinetics of firefly luciferase. When firefly luciferase is mixed with luciferin and ATP, a transient flash of light is produced, followed by a weak light, lasting hours. The first stage reaction produces an enzyme-luciferin-AMP complex and pyrophosphate. Addition of pyrophosphate to the reaction mixture decelerated the reaction rate, and the initial flash was prolonged to a plateau, showing a quasi-equilibrium state. The effects of temperature and pressure were analyzed at the plateau. The temperature scan showed that the maximum light intensity was observed at about 22.5°C. When pressurized below the temperature optimum, pressure decreased the light intensity, while increasing it above the temperature optimum. According to the theory of absolute reaction rate, the following values were obtained for the bioluminescent reaction: $\Delta V^{\ddagger} = 823.7 - 2.8T$ cm^3/mol and $\Delta V = -280.47 + 0.94T$ cm^3/mol, where T is the absolute temperature, $\Delta V^{\ddagger}$ and ΔV are, respectively, activation volume and the volume change due to thermal unfolding. The optimal temperature for the maximum light output occurs because the reaction rate increases with the temperature elevation at low temperature range, but the thermal unfolding of the enzyme decelerates the reaction velocity when the temperature exceeds a critical value. The intensity of luminescence is modified by the influence of pressure on both $\Delta V^{\ddagger}$ and ΔV. So long as the volume of the activated complex ($V^{\ddagger}$) exceeds the average volume of the nonactivated complex (V_N), pressure will slow down the reaction. At the point where the volumes become equal, there is no change in the rate under pressure. When the volume of the activated complex is less than that of the reactants, pressure will speed up the rate. This study showed that firefly luciferase is not exceptional to other enzymes in responding to high pressure.

INTRODUCTION

High pressure antagonizes anesthetic actions. The pressure reversal of anesthesia was discovered by Johnson and co-workers (1942) in the light intensity of luminous bacteria. From the effects of temperature and pressure on the bacterial luminescence, Eyring and Magee (1942) analyzed the molecular mechanism of pressure reversal. The criticism that bacterial luminescence has no relevance to anesthesia was dealt with by Johnson and Flagler (1951) by demonstrating that tadpoles, anesthetized with ethanol, started swimming again when hydrostatic pressure in the range of 100 atm was applied.

Against the general contention that pressure always antagonizes anesthesia, Moss et al. (1991) reported that pressure did not show any effect on the initial flash intensity of the purified lipid-free firefly luciferase in the presence or absence of anesthetics. This is peculiar because reaction rate is a function of temperature and pressure.

Firefly luciferase emits flash of light when mixed with luciferin and ATP in the presence of oxygen. The initial flash of light is followed by a weak light intensity with a slow decline continuing for hours. The reaction sequence of light emission was clarified by McElroy and co-workers (McElroy and Seliger, 1962; McElroy et al., 1969) as follows:

$$E + LH_2 + ATP = E \cdot LH_2 \cdot AMP + PPi \qquad (1)$$

$$E \cdot LH_2 \cdot AMP + O_2 \rightarrow E \cdot L_{ox} \cdot AMP + CO_2 + Light$$

where E is the firefly luciferase, LH_2 is the reduced form of luciferin, L_{ox} is oxiluciferin, and PPi is inorganic pyrophosphate. When pyrophosphate was added to the system, the reaction rate was decelerated by the product inhibition, and the initial flash peak was transformed into a plateau. This study analyzed the pressure effect on the light intensity measured in the presence of pyrophosphate.

MATERIALS AND METHODS

Lyophilized crystalline firefly luciferase, synthetic D-luciferin, Na_2-ATP, tetrasodium pyrophosphate decahydrate, and glycylglycine were obtained from Sigma (St. Louis, MO), and $MgCl_2$ from Baker (Phillipsburg, NJ). Water was purified by distillation followed by purification with a Sybron/Barnstead system (Boston, MA) consisting of two mixed-bed ion-exchanger columns, an activated charcoal column, and an ultrafilter.

The initial flash intensity at ambient pressure was measured by a Durrum model D-110 pneumatic-drive stopped-flow spectrophotometer (Sunnyvale, CA) with a 99.5% mixing time of 2 ms. Luciferase was dissolved in a 100 mM glycylglycine buffer (pH 7.8) at a concentration of $1.0 \cdot 10^{-2}$ mg/1.0 ml buffer together with luciferin 0.1 μmol. The ATP mixture contained 0.6 mM Na_2-ATP and 10 mM $MgSO_4$ in the same buffer. Each solution was loaded in a 5-ml syringe. The luciferin-luciferase solution was mixed with the ATP solution at a mixing ratio 1:1 by volume. Nitrogen gas with 10 kg/cm^2 pressure was used to mix the contents of two syringes. The temperature of

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the sample, controlled by circulating water around the system from a constant temperature water bath, was maintained at ±0.5°C of the desired temperature. The light intensity was measured by a photomultiplier. The photomultiplier output was monitored by a Nicolet 310 digital recording oscilloscope (Madison, WI) and stored on a floppy disk.

The pressure effect in the presence of pyrophosphate was measured with an AMINCO high-pressure cell with sapphire windows (Silver Spring, MD). The sample compartment of a Hitachi Perkin-Elmer model 139 spectrophotometer (Norwalk, CT) was replaced by the high-pressure cell assembly. The high-pressure cell was covered by a water jacket, and the temperature of the cell was controlled by circulating water from a water bath. The cuvette temperature was monitored by a filament thermistor inserted into a hole of the pressure block and a Digitec thermometer (United Systems, Dayton, OH) with 0.01°C resolution. Luciferase was rapidly mixed with luciferin and ATP in a 20-ml syringe and injected into the pressure cuvette immediately after mixing.

Pressure was scanned by a custom-fabricated motor-driven hydraulic pump connected to the pressure cuvette by a stainless steel tube via a liquid separator. The pump and the connecting tube were filled with hexane. Compression to 400 atm was completed within 2 min. Pressure was measured with an accuracy of ±1 psi (0.07 bar) by an Autoclave Engineers pressure transducer system, model DPS-0201 (Erie, PA). The photomultiplier output was monitored by the Nicolet 310 digital recording oscilloscope and was stored on a floppy disk with the signal from the pressure transducer.

The effect of temperature on the light intensity in the presence of pyrophosphate at ambient pressure was measured by the Hitachi Perkin-Elmer 139 spectrophotometer with the light source turned off. The temperature was scanned between 10 and 35°C by circulating water from a computer-controlled Haake F3-CH water bath (Berlin, Germany). The temperature of the reaction mixture was measured by the Digitec thermistor thermometer. The thermistor tip was inserted into the cuvette. The reaction mixture was continuously mixed with a direct-drive stirrer (Spectrocell, Oreland, PA) to ensure the homogeneous temperature distribution. The temperature scan was completed within 2 min. The thermometer output was monitored by the Nicolet 310 digital recording oscilloscope together with the output from the photomultiplier and stored on a floppy disk.

RESULTS

Fig. 1 shows the effect of the pyrophosphate/ATP mole ratio on the light intensity by taking the ratio between the light intensities at 60 s after the mixing and the initial light intensity. From the curve, the ATP/pyrophosphate concentration ratio was selected to be 1:10. Fig. 2 shows the light intensity in the presence of 3 mM pyrophosphate at 23°C measured at ambient pressure by the stopped-flow system. The light intensity was almost parallel to the time axis, indicating that the system was in a quasi-equilibrium state. A slow decay of the light intensity was observable after 2 min.

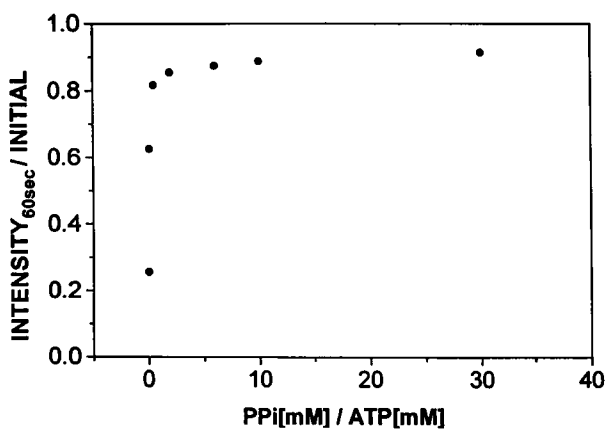

FIGURE 1 Effect of pyrophosphate concentration on the initial flash intensity of luciferase. Abscissa is plotted by the pyrophosphate/ATP concentration ratio. ATP concentration was 0.3 mM. Ordinate is the ratio between the light intensity at 60 s after the mixing and the initial light intensity. The light intensity was measured by the voltage generated by the photomultiplier.

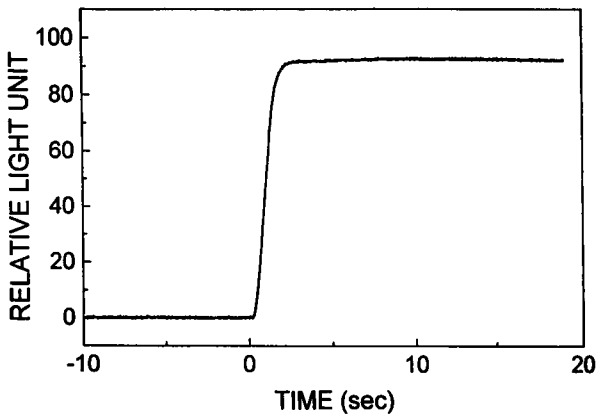

FIGURE 2 Stopped-flow tracing of light intensity in the presence of 3.0 mM pyrophosphate. The light output is almost parallel to the time axis.

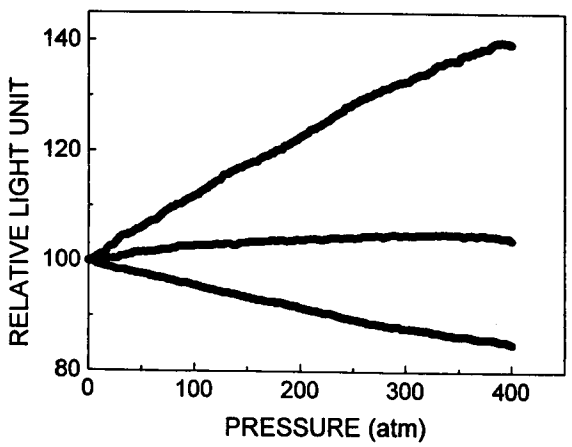

FIGURE 3 Pressure scan of the light output. Pressure was scanned by a motor-driven hydraulic pump. The temperatures were from the top: 30, 22.5, and 17.5°C. At high temperatures, the light intensity increased, and at low temperatures, it decreased. At 22.5°C, high pressure showed little effect. The light intensity is expressed by the percent of the control at zero applied pressure.

Fig. 3 shows the raw data on the pressure scan up to 400 atm. The light intensity increased when compressed at high temperature (30°C) and decreased at low temperature (17.5°C). At 22.5°C, compression did not change the intensity appreciably.

The optimal temperature for the maximal light output in the presence of pyrophosphate was measured at ambient pressure by scanning the temperature and is shown in Fig. 4. The maximum light intensity was observed at 22.5°C.

Fig. 5 shows the effect of high pressure on the light intensity at various temperatures constructed by pooling all data. At least three studies were performed at each temperature. The temperatures are from the top: 32.0, 30.0, 27.0, 22.5, 17.5, and 15.0°C.

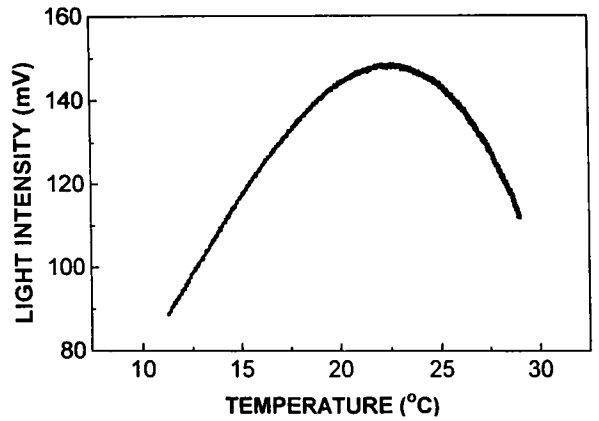

FIGURE 4 Temperature scan of the light intensity in the presence of pyrophosphate. Temperature was scanned by circulating water from the computer-controlled water bath. The maximal light output was observed at about 22.5°C.

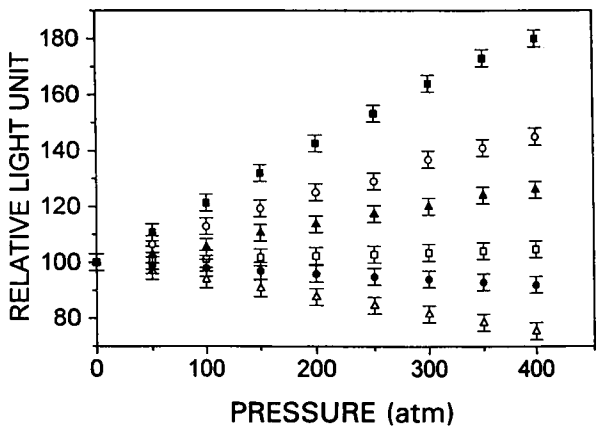

FIGURE 5 The pressure effects on the light intensity at various temperatures. The temperatures are from the top: 32.0, 30.0, 27.0, 22.5, 17.5, and 15.0°C. The light intensity is expressed by the percent of the control at zero applied pressure. The data points were the average of at least three experiments with standard error bars.

The light intensity decreased when the system was compressed at temperatures below the optimum and increased when the system was compressed at temperatures above the optimum. At the optimal temperature, high pressure did not affect the light intensity.

DISCUSSION

The absence of pressure effects on the purified lipid-free firefly luciferase, reported by Moss et al. (1991), appears to indicate that the pressure reversal of anesthesia is caused by the pressure effect solely on the lipid part of the excitable system; proteins are not involved. The present study, however, showed that the purified lipid-free firefly luciferase responds to high pressure in a biphasic mode: inhibition at low temperature and activation at high temperature.

The temperature-dependent opposite effect of pressure on enzyme kinetics is analyzed according to the rate theory (Neville and Eyring, 1972). At the present experimental condition where the system is in a quasi-equilibrium state, we assume that the overall reaction leading to light emission proceeds as if governed by a single specific-reaction rate constant k'.

In the presence of excess luciferin, the light intensity, I, is proportional to the concentration of luciferase [E] and [ATP] with proportionality constant b.

$$I \propto V = bk'[\mathrm{E}][\mathrm{ATP}] \tag{2}$$

It is also a function of the state of luciferase, expressed by the equilibrium constant, K, between the N-state (folded conformation, active) and the D-state (unfolded conformation, inactive).

$$I = \frac{bk'[\mathrm{ATP}][\mathrm{E}]}{1 + K} \tag{3}$$

According to the theory of absolute reaction rate,

$$k' = \kappa\left(\frac{kT}{h}\right)K^{\ddagger} = \kappa\left(\frac{kT}{h}\right)\exp\left(-\frac{\Delta G^{\ddagger}}{RT}\right) \tag{4}$$

where k is Boltzmann's constant, h is Planck's constant, and κ is the transmission coefficient, which is often unity. The light intensity is now expressed as

$$I = \frac{b\kappa(kT/h)K^{\ddagger}[\mathrm{ATP}][\mathrm{E}]}{1 + K} \tag{5}$$

and

$$\Delta G = \Delta H_0 - T\Delta S_0 + p\Delta V,$$

and (6)

$$\Delta G^{\ddagger} = \Delta H_0^{\ddagger} - T\Delta S_0^{\ddagger} + p\Delta V^{\ddagger}$$

where subscript 0 indicates zero applied pressure (see Appendix for derivation of the equation). Then,

$$I = \frac{cT\exp(-\Delta H_0^{\ddagger}/RT)\exp(-p\Delta V^{\ddagger}/RT)\exp(\Delta S_0^{\ddagger}/R)}{1 + \exp(-\Delta H_0/RT)\exp(-p\Delta V/RT)\exp(\Delta S_0/R)} \tag{7}$$

where all constants, $b\kappa(k/h)[\mathrm{ATP}][\mathrm{E}]$, are put together and expressed by c.

At temperatures well below the optimum, the volume associated with thermal unfolding may be negligibly small in comparison to unity and so has virtually no influence on the rate. The effects of increased pressure then occur practically entirely through changes in the specific reaction-rate constant. At temperatures higher than optimal, the intensity of luminescence is modified by the influence of pressure on both $\Delta V^{\ddagger}$ and ΔV. The numerical values were obtained, first estimating $\Delta V^{\ddagger}$ at low temperatures (12–15°C), and ΔV is estimated by subtracting the $\Delta V^{\ddagger}$ from the value obtained at high temperatures (30–33°C). We obtained $\Delta V^{\ddagger} = 823.7 - 2.8T$ cm^3/mol and $\Delta V = -280.47 + 0.94T$ cm^3/mol.

The rate process measures the concentration of activated complex because the complexes always decompose at the same rate, kT/h (Neville and Eyring, 1972). The activated

complex is in equilibrium with its environment and is composed of a species whose heat content is changing with temperature. When the activated complex represents a configuration more energy-rich than nonactivated complex, the temperature elevation increases the rate. At the point where the two are the same, the rate reaches a maximum. At still higher temperatures, the reversibly inactivated D-state enzyme has more energy-rich configurations. The temperature elevation decreases the rate. An exactly parallel situation exists with respect to pressure. So long as the volume of the activated complex ($V^{\ddagger}$) exceeds the average volume of the nonactivated complex (V_N), pressure will slow down the reaction. At the point where the volumes become equal, there is no change in the rate under pressure. When the volume of the activated complex is less than that of the reactants, pressure will speed up the rate.

The negative result reported by Moss et al. (1991) gives an idea that protein functions are not affected by moderate pressures. Nevertheless, aside from the present demonstration, there are ample reports on the pressure effects on the lipid-free enzyme systems, for instance, lysozyme (Morild, 1977), alcohol dehydrogenase (Carter et al., 1978), ribonuclease (Taniguchi and Suzuki, 1983), chymotrypsin (Frauenfelder et al., 1990), myoglobin oxygen dissociation (Weber and Drickamer, 1983), and a review (Johnson et al., 1974).

The failure to recognize a pressure effect (Moss et al., 1991) may indicate that the initial flash intensity is insensitive to high pressure or may be due to their choice of the temperature. Their pressure study was performed at ambient temperature, which is near the maximum reaction velocity. The present study showed that pressure has negligible effects on the light intensity at 20–25°C.

The pressure effect must be evaluated at a steady state (Neville and Eyring, 1972). The kinetics of initial burst of light and the secondary low luminescence is complex and the assignment of steady state is unclear. The lack of response of the initial flash intensity to high pressure may indicate that the initial peak may not represent the steady state. We found that the secondary low-intensity luminescence responds to pressure similar to the present study. The low level intensity increased when pressurized at the high temperature range and decreased at the low temperature range. The finding appears to indicate that the secondary part may be closer to the steady state where the concentrations of all members of reactants stay stationary. Whatever the kinetics may be, pressure affected the light intensity, depending upon the temperature in this one-enzyme three-substrate system.

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APPENDIX

In thermodynamics, the free energy is described either by constant temperature or by constant pressure. The following is the derivation of Eq. 6.

$$G(T, P) = H - TS$$

$$\Delta G(T, P)^{T}_{p\to 0}$$

$$= \Delta(H - TS)^{T}_{p\to 0}$$

$$= \Delta\left\{(H_0 - TS_0)^{T}_{p=p_0} + \left(\frac{\partial(H - TS)}{\partial p}\right)^{T}_{p=p_0}\Delta p\right\}$$

$$= \Delta H^{T}_0 - T\Delta S^{T}_0 + \Delta\left\{\left(\frac{\partial(H - TS)}{\partial p}\right)^{T}_{p=p_0}\Delta p\right\}$$

$$= \Delta H^{T}_0 - T\Delta S^{T}_0 + \Delta\left\{\left(V - T\left(\frac{\partial V}{\partial T}\right)_{p=p_0}\right) + T\left(\frac{\partial V}{\partial T}\right)_{p=p_0}\right\}\Delta p$$

$$= \Delta H^{T}_0 - T\Delta S^{T}_0 + \Delta V \cdot \Delta p = \Delta H^{T}_0 - T\Delta S^{T}_0 + p\Delta V$$

$$(\because \quad \Delta p = p - p_0; \quad p_0 = 0)$$

For this reason, subscript 0 is added to Eq. 6 and subsequent equations to indicate zero applied pressure.

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