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Ethanol unfolds firefly luciferase while competitive inhibitors antagonize unfolding: DSC and FTIR analyses*

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Abstract: Firefly luciferase has gained popularity as a protein model in elucidating anaesthesia mechanism because the bioluminescence of the purified enzyme system is extremely sensitive to volatile anaesthetics. This study analysed the thermal unfolding of firefly luciferase by differential scanning calorimetry (DSC) and Fourier transform infrared spectroscopy (FTIR). DSC showed that the transition of firefly luciferase from the folded (N) to unfolded (D) state occurred at 41.7°C with the excess heat flow of 1.6 cal g^{-1} protein. Ethanol decreased the transition temperature dose dependently. In contrast, luciferin competitors, anilinonaphthalenesulphonate (ANS), toluidinonaphthalenesulphonate (TNS), and myristic acid increased the transition temperature. The competitive inhibitors antagonized unfolding and stabilized the N-state. Ethanol promoted unfolding and stabilized the D-state. Temperature scan by FTIR agreed with the DSC data. The intensities of amide-I′ and amide-II′ bands started to increase at 20–25°C. This temperature coincides with the temperature where the bioluminescence of firefly luciferase is maximal. The unfolding effect of ethanol was evident even at 5°C. ANS, TNS, and myristic acid completely protected the enzyme from the thermal unfolding. This is the first demonstration that the noncompetitive inhibitors induce the isothermal first-order phase transition in a functional protein, whereas competitive inhibitors protect the enzyme from thermal unfolding. The action mode of competitive inhibitors on firefly luciferase is completely different from that of noncompetitive inhibitors.

Keywords: *Anaesthesia theory; alcohols; protein folding; enzyme protein; noncompetitive inhibitor; protein phase transition; calorimetry.*

Introduction

Despite the discovery of anaesthesia more than a century ago, its mechanism defied clarification. There is a controversy on the site of action of anaesthetics between lipid membranes and proteins. The favour for lipids as the anaesthetic action site, based on the Meyer–Overton theory, appears to have shifted to proteins in recent years. Protein theories of anaesthesia are divided in two: those which assume the presence of specific binding sites for uncharged anaesthetics and those which assume nonspecific conformational change of proteins. We contend that anaesthetics interact with lipid membranes and proteins indiscriminately. This concept is unconventional because many believe either lipids or proteins are the site of action of anaesthetics. Compared to a large number of reports on lipid–anaesthetic interaction, reports on protein–anaesthetic interaction are few. This is because purified lipid-free enzymes are not very sensitive to anaesthetics.

In 1965, Ueda reported that the ATP-induced bioluminescence of cell-free preparation of firefly luciferase is inhibited by volatile anaesthetics even at subclinical concentrations [1]. Firefly lanterns contain an enzyme luciferase and its substrate luciferin. The purified enzyme–luciferin mixture emits light when ATP is added. The light intensity is proportional to the added amount of ATP. Since the ATP-specificity is so rigid, the system is used to quantify ATP content in biological materials. Firefly luciferase has been extensively investigated by McElroy and coworkers [2] and the enzyme was identified as a hydrophobic protein consisting of two 50 kDa subunits [3].

Subsequently, Ueda and Kamaya [4] reported that the inhibition is allosteric, and

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accompanied by conformational change (unfolding) of the enzyme. This was estimated by the temperature-dependence of the inhibitory effect of volatile anaesthetics on the bioluminescence of firefly luciferase, and applying the Eyring theory of absolute reaction rate. Franks and Lieb [5, 6], however, reported that volatile anaesthetics inhibited firefly luciferase by competition to the substrate luciferin. The competition was proposed by the kinetic analysis of inhibitory action of anaesthetics on the bioluminescence of firefly luciferase with the Lineweaver–Burk graphical method. It is well known that the double reciprocal plot often fails to distinguish between competitive and noncompetitive inhibitions [7].

DeLuca [8] reported that anilinonaphthalenesulphonate (ANS) and toluidinonaphthalenesulphonate (TNS) are strong luciferin competitors. This was demonstrated not only by the conventional graphical analysis of the inhibitory effects, but with displacement of these chromophores from the enzyme surface by the substrate luciferin.

To re-evaluate our previous conclusion, this study measured conformational change of luciferase by direct methods, and compared the effects of ethanol with the chromophores of luciferin binding. We found that long-chain fatty acids are also a strong luciferin competitor (to be reported).

Differential scanning calorimetry (DSC) and Fourier transform infrared spectroscopy (FTIR) were used to examine the effects of ethanol on the thermotropic phase transition of crystalline firefly luciferase. The result was compared with the effect of ANS.

Methods

Lyophilized crystalline firefly luciferase, D-luciferin, myristic acid, and glycylglycine were obtained from Sigma, magnesium 8-anilino-1-naphthalenesulphonate (ANS), potassium 6-p-toluidino-2-naphthalenesulphonate (TNS) and chloroform from Baker (Phillipsburg, NJ, USA), ethanol from US Industrial Chemicals (Anaheim, CA, USA). Deuterated ethanol (ethanol-d_5), deuterium oxide (D_2O), and deuterated glycine (glycine-d_5) were obtained from Sigma, and NaOD from Fluka (Ronkonkoma, NY, USA).

Phase transition was measured by a MicroCal MC2 differential scanning calorimeter (Northampton, MA, USA), interfaced with an 80486 personal computer. Crystalline luciferase was dissolved in 100 mM glycylglycine buffer pH 7.8 at a concentration of 0.3% (w/v) and loaded into the reaction chamber of 1.4 cm^3 capacity. The reaction cell was pressurized by nitrogen at 1.2 atm. The heating rate was 10°C h^{-1} between 4°C and 70°C. The DSC thermograms were analysed by MicroCal ORIGIN software.

The structural change of luciferase was measured by a Perkin–Elmer (Norwalk, CT, USA) Model 1750 Fourier transform infrared spectrophotometer (FTIR) interfaced with an 80486 personal computer. The data were analysed by Perkin–Elmer IR Data Manager software. Luciferase was dissolved in D_2O with 100 mM glycine-d_5 buffer pH 7.8 at a concentration of 0.6% (w/v) and loaded into a high-pressure attenuated total reflectance (ATR) CIRCLE cell with a zinc selenide window (0005-302, Spectra–Tech, Stamford, CT, USA). A triglycine sulphate detector was used. Each sample was scanned 40 times through the frequency range of 700–4000 cm^{-1} and averaged. The spectra of the solvents were subtracted from the spectra of the solution. The spectra were analysed by Perkin–Elmer IR Data Manager. The resolution enhancement was performed by Deconvolution Function routine of Data Manager by using a Lorenzian bandwidth of 18 cm^{-1}.

Results

DSC showed that thermal phase transition occurred with transition midpoint, T_M, at 41.7°C with the excess heat flow of 1.6 cal g^{-1} protein (Fig. 1). Ethanol decreased the phase-transition temperature dose-dependently. In contrast, addition of 1 mM luciferin increased the transition temperature. Addition of 0.1 mM ANS, TNS, or myristic acid also increased the transition temperature. In the presence of these competitive inhibitors, excess heat flow was not observed when the temperature was scanned even up to 90°C (not shown). Figure 1 shows the dose-dependent decrease of the phase transition temperature by ethanol. The excess heat flow was not observed during the cooling scan, suggesting that the temperature for the irreversible aggregation may not be very far from the N–D transition.

Our experience with the thermal phase transition of homopolymer polypeptide, poly(L-lysine), from α-helix to β-structure

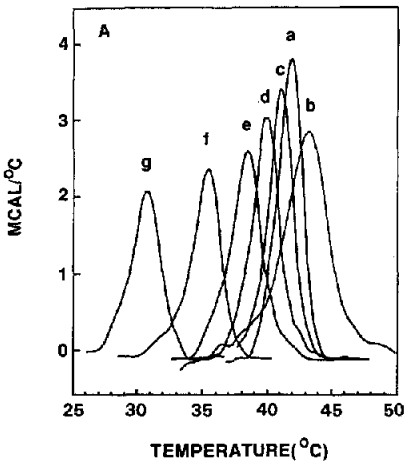

Figure 1
DSC thermograms of the effects of ethanol on the thermal phase transition of firefly luciferase. The dose-dependent ethanol effects. Signs are: (a) control thermogram (T_M = 41.7°C), (b) luciferin 1 mM (43.1°C), (c) ethanol 0.15 M (40.8°C), (d) 0.3 M (39.6°C), (e) 0.6 M (38.2°C), (f) 1.2 M (35.2°C) and (g) 2.3 M (30.6°C).

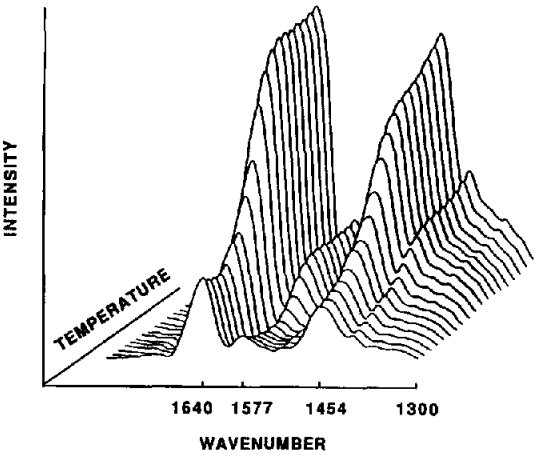

Figure 2
FTIR spectra showing thermal conformational change of firefly luciferase. The peak at 1640 cm^{-1} represents Amide-I', 1577 cm^{-1} Amide-II and 1454 cm^{-1} Amide-II'. The protein conformational change is seen by the jump in the spectral intensity starting at 20–25°C and ending at 48°C. The temperatures was scanned between 5 and 70°C.

also showed no excess heat flow during the cooling scan of poly(L-lysine) [9–11]. Nevertheless, the transition is not absolutely irreversible, because incubation of the β-structure peptide under pressure in cold for several days resulted in partial recovery of α-helix structure [11]. The reversibility of the thermal unfolding of firefly luciferase is uncertain. Incubation of the unfolded enzyme in a refrigerator for 48 h did not show any excess heat flow by DSC.

The temperature-induced conformational changes of firefly luciferase were analysed by FTIR. Figure 2 is the amide-I' and II' region of the temperature scan of firefly luciferase dissolved in D_2O. The peak at 1640 cm^{-1} represents the Amide-I' band. The Amide-II' band was split into two: 1577 cm^{-1} and 1454 cm^{-1}, due to the deuteron–proton exchange of amino acid residues located at the surface of the proteins. Those not deuterated appeared at 1577 cm^{-1} (Amide-II), and those deuterated appeared at 1454 cm^{-1} (Amide-II'). The thermotrophic conformational change is clearly observable in these FTIR spectra by the jump in the spectral intensity starting at 20–25°C and ending at about 48°C.

In the presence of 0.1 mM ANS, TNS, or myristic acid, the temperature-induced change in the secondary structure of the enzyme protein completely disappeared. The transition was not observed even at 70°C. It has long been known that long-chain fatty acids bind to bovine serum albumin and protect the protein from thermal unfolding [12–14]. In contrast, 1.5 M *d*-ethanol enhanced the thermal effects on the spectral intensity. In Fig. 3, the effects of 1.0 mM ANS and 1.5 M *d*-ethanol on the peak heights of Amide-I' (Fig. 3a) and II' (Fig. 3b) are plotted against the temperature. The controls are plotted by closed circles, ANS by open circles, and ethanol by open triangles. Notice that in the presence of ethanol, the intensities of all peaks are stronger than the control even at 5°C.

To evaluate the ethanol effect on the protein structure, the FTIR data were analysed by the Resolution Enhancement routine of Perkin–Elmer IR Data Manager. Figure 4 shows the resolution-enhanced spectra.

Figure 4(a) is a control without inhibitors. Elevation of the temperature increased the 1624 cm^{-1} peak intensity, and decreased the

Table 1
Effect of ethanol on the phase transition temperature, T_M, of firefly luciferase

		Effects of ethanol				
Concentration (M)	Control	0.15	0.30	0.60	1.20	2.30
Temperature (°C)	41.7	40.8	39.6	38.2	35.2	30.6

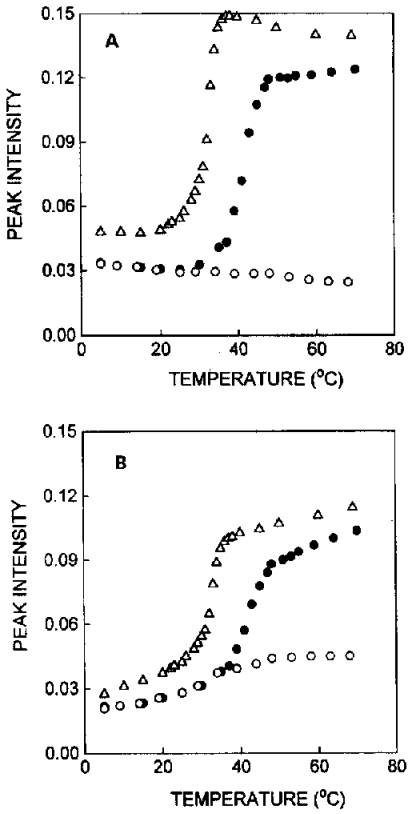

Figure 3
Effects of ethanol and a competitive inhibitor, ANS, on the spectral intensity of Amide-I′ (A) and Amide-II′ (B) peaks. Symbols are: control (closed circles), *d*-ethanol 1.5 M (open triangles), and ANS 0.1 mM (open circles). The competitive inhibitor prevented the thermal change of luciferase, while ethanol enhanced it.

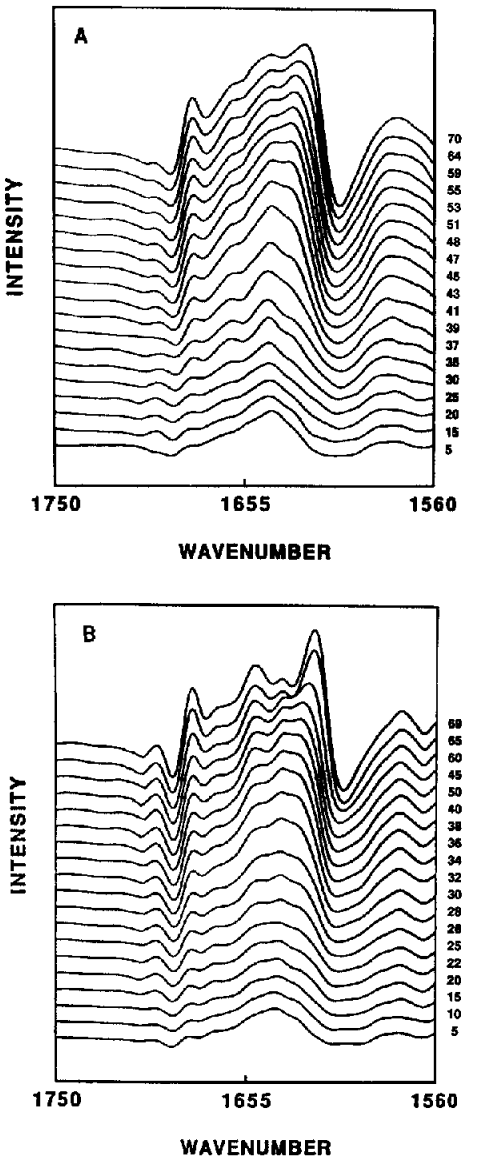

Figure 4
Enhanced FTIR spectra between 1560–1759 cm^{-1}: (A) control and (B) with 1.5 M *d*-ethanol. The scanning temperatures are shown at the right side in °C. The peaks are: 1624 cm^{-1} β-sheet, 1639 cm^{-1} possibly α-helix, 1680 cm^{-1} anti-parallel β-sheet and 1660 and 1700 cm^{-1} β-turns (14).

1639 cm^{-1} intensity. Figure 4(b) shows that ethanol amplified the change in the increase in the 1624 cm^{-1} intensity and the decrease in the 1639 cm^{-1} intensity. The 1624 cm^{-1} peak represents β-sheet [15], and the 1639 cm^{-1} peak probably represents α-helix. The 1639 cm^{-1} peak is lower than the usual position of α-helix peak in many proteins, where α-helix appears at 1650–1660 cm^{-1} region [16]. The cause of this low frequency shift is unclear. Possibly, luciferase may contain higher degree of regularity, such as inclusion of α_{10} or 3_{11} helices [17], or it may include random-coil. The dose-dependent effect of ethanol on the thermotropic conformational change was expressed by the ratio of the intensities between the 1639 cm^{-1} and 1624 cm^{-1} peaks and is shown in Fig. 5.

Ethanol induced protein conformational change at much lower temperature. The 1624 cm^{-1} peak gradually shifted to 1615 cm^{-1} as the temperature increased. At the highest temperature, the 1615 cm^{-1} peak predominated, which is characteristic of aggregation. The unfolding of protein means that the hydrophobic interior is exposed to the aqueous phase, which probably facilitates aggregation.

Discussion

With direct methods, the present study demonstrated unambiguously that ethanol inhibits firefly luciferase by conformational

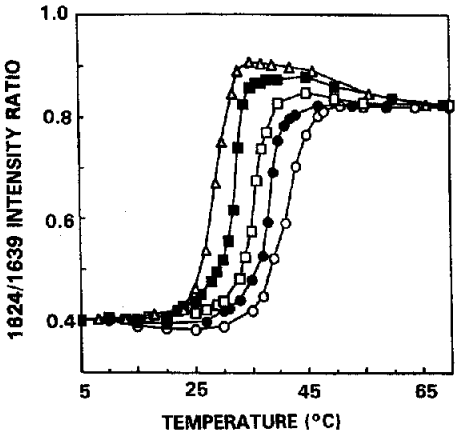

Figure 5
Effect of *d*-ethanol on the luciferase conformational change. The heat-induced conformational change is expressed by the ratio of the spectral intensities between 1624 cm^{-1} (β-sheet) and 1639 cm^{-1} (α-helix). Ethanol uncoiled the α-helix dose dependently. The symbols are: control without *d*-ethanol (open circles), *d*-ethanol 0.5 M (closed circles), 1.0 M (open squares), 1.5 M (closed squares) and 2.0 M (open triangles).

change of the enzyme protein. The luciferin competitors did not change the protein conformation. It confirms our previous conclusion [4], and contradicts the reports of Franks and Lieb [5], who proposed that anaesthetics inhibit firefly luciferase by competing with the substrate luciferin.

Luciferin is a charged hydrophobic molecule. It is difficult to see that anaesthetic molecules without ionic charge can locate the luciferin recognition site on the enzyme. Anaesthetics and alcohols bind all hydrophobic areas on the protein surface and change the protein conformation. There appears to be a concept that alcohols are not an anaesthetic. However, clinical anaesthesiologists know that intravenous infusion of ethanol solution induces anaesthesia.

The conformational change, however, damages luciferin recognition site and interferes with the substrate binding. DeLuca [8] reported that incubation of the enzyme with sulphhydryl agents, such as *N*-ethylmaleimide, inhibited bioluminescent activity and decreased the affinity of the probe dye, TNS, which binds to the luciferin recognition site.

Depression of the phase-transition temperature by anaesthetics is analogous to the depression of the freezing point of water by adding salt. Salt melts ice, because salt molecules dissolve only into water and do not dissolve in ice. If salt molecules dissolve in ice (low temperature state) and not in water (high temperature state), the freezing point should increase.

Proteins change their conformation by heat or by chemical denaturants, such as urea and guanidine. The conformational change is designated as unfolding from the native folded state (N-state) to the denatured unfolded state (D-state). Thus, enzyme proteins exist at least in two states. According to the above model of freezing temperature depression (van't Hoff model), the decrease in the phase-transition temperature by anaesthetics means that these molecules preferentially bind to the high temperature D-state of the protein. On the other hand, the competitive inhibitors, ANS, TNS and myristate, preferentially bind to the low temperature N-state. It has been amply demonstrated that anaesthetics decrease the phase transition temperature of lipid membranes between solid-gel and liquid-crystalline phases [19]. The present study shows that anaesthetics affect proteins and lipid membranes similarly.

FTIR showed that the thermal change in the protein structure started at 20–25°C. This is the temperature where the light intensity is maximal [1–7]. The decline in the enzyme reaction rate above this temperature corresponded to the start of the thermal unfolding of the enzyme protein shown by FTIR. The agreement between the optimal temperature for bioluminescence and the temperature where FTIR spectra increase their intensity indicates that the thermal unfolding inhibits the enzyme activity. The conformational change, which is observed by FTIR in lower temperatures, was not the first-order phase transition, as shown by the DSC study.

Below 20°C, the control FTIR spectra did not change significantly, whereas ethanol affected the spectra at 5°C (Fig. 3). The inhibitory effect of ethanol on luciferase luminescence is attributable to the conformational change of the enzyme protein. The competitive inhibitors prevented the enzyme protein from thermal unfolding. The inhibitory effects of competitive inhibitors depend solely on interfering with the luciferin binding.

The term 'protein unfolding', however, has not been clearly defined. By differential scanning calorimetry, unfolding is envisioned as a highly cooperative phenomenon between the folded (native) and unfolded (denatured) phases: proteins exist either in native or de-

natured forms in an all-or-none mode [20]. Protein folding attracted recent researches since the discovery of chaperonin, which guides newly synthesized strings of amino acids to a biologically meaningful globular conformation [21]. Folding occurs through the 'molten globular' state. The molten globular state is said to be the intermediate between the native and denatured states [22]. 'Partial unfolding' is the term used to describe the protein structure in this state. Molten globule is depicted by the secondary structure similar to the unfolded with different tertiary structure. Accordingly, folded, unfolded, intermediate, and molten globule states are now used with limited meaning.

When unfolding is designated as the all-or-none first-order phase transition, the anaesthetized luciferase with decreased activity cannot be described as partially unfolded state. The FTIR study, however, showed a gradual change in the secondary structure when the temperature was raised before demonstrating the N–D transition. Shimizu *et al.* [23] reported that unfolding of the molten globule state is not a two-state process. The anaesthetized state is intermediate between less-active and more-active states. Firefly luciferase exists as an intermediate state at temperatures below 41°C, fluctuating rapidly between low-temperature and high-temperature states. In a macroscopic model, the intermediate state can be envisioned as an equilibrium state between the two. Anaesthetics shift the equilibrium to the high-temperature state, while competitive inhibitors shift it to the low-temperature state.

Firefly luciferase is unique among purified enzymes in that the action is highly susceptible to inhibition by anaesthetics. Almost all cell-free enzymes are resistant to anaesthetics except in a great excess of clinical concentrations. These include cytoplasmic glycolytic enzymes and mitochondrial electron transport enzymes [24]. The expectation that membrane enzymes, such as $(Na^+ + K^+)$-ATPase, may be susceptible to anaesthetics was failed. The inhibitory effect on ouabain-sensitive $(Na^+ + K^+)$-ATPase was demonstrable only when the anaesthetic concentration was increased about one order of magnitude larger than the clinical concentrations [25]. It is interesting to note that other light emitting enzymes, such as bacterial luciferase, are also highly susceptible to anaesthetic inhibition [26–29].

Due to the high sensitivity of firefly luciferase, the enzyme is advocated to be a good model to analyse the mode of anaesthetic interaction with proteins. We emphasize that the system is a protein model. Consequently, the phase-transition temperature of 41.7°C has no meaning to the activity of fireflies because they may not survive this temperature. It is important only to elucidate the mode of anaesthetic–protein interaction.

Model systems are often the target of criticism with regard to their relevance to biological functions. When the antagonistic action of high pressure against anaesthetics was first reported by Johnson, Eyring and co-workers in 1942 [26, 27], the study was criticized on the grounds that bacterial bioluminescence has no relevance to brain functions. The criticism was dealt with by Johnson and Flagler [30] 10 years later with the demonstration that tadpoles anaesthetized by ethanol instantly started swimming again when pressurized.

Conclusion

The present result supports the Erying model of enzyme inhibition [17], where inhibitors are classified as two types: those which facilitate thermal unfolding, and those which compete with substrates without inducing unfolding. As we concluded previously [4], ethanol unfolded the enzyme protein. According to the van't Hoff model of freezing temperature depression, the decrease in the phase transition temperature by ethanol indicates that these molecules preferentially bind to the unfolded state of the protein. The competitive inhibitors preferentially bound to the folded state and stabilized it. The possibility of anaesthetics competing with neurotransmitters at the binding site is remote, because molecules that bind to the native state (intact binding site) increase the transition temperature.

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Appendix

In the resolution enhancement spectra, the 1639 cm^{-1} peak is at lower frequency than the usual position of many proteins, where the α-helix appears at about 1650–1660 cm^{-1} region. The exact cause of this low frequency is unclear. Possible contributing factors include that luciferase may contain higher degree of regularity, effect of β-structure and random coil, etc. Perhaps, there are some influences caused by α_{11} or 3_{10} helix. These helix shows significantly stronger peak intensity. Other possibilities are; the peak contains β-structure, and the tail of the β-structure band at 1624 cm^{-1} is affecting the 1639 cm^{-1} intensity.

With poly(L-lysine), α-helix peak appears at 1638 cm^{-1}. Anaesthetics partially transformed α-helix poly(L-lysine) to β-structure [8–10]. For this reason, the decrease in the ratio between 1639/1624 by anaesthetics suggests that anaesthetics increased β-structure in the expense of α-helix.

The bands at 1577 cm^{-1} and 1454 cm^{-1} correspond to the COO^- and CONH vibration, respectively. The COO^- is easy to undergo proton–deuteron exchange, while CONH requires longer time. If an H–D exchange occurs, the band at 1639 cm^{-1} will decrease and the band at 1577 or 1454 cm^{-1} will increase. (The ratio 1639/1577 and 1639/1454 should decrease.) However, our results in Fig. 3 show that the ratio 1639/1577 or 1639/1454 did not change at various temperatures. Under this condition, the deuteron–proton exchange is completed in COO^-, but not in CONH.