Cypridina Bioluminescence: Light-Emitting Oxyluciferin-Luciferase Complex

Abstract. *Fluorescence of* Cypridina *oxyluciferin is greatly enhanced when it is bound to luciferase; the spectrum is thereby shifted, so that it corresponds precisely to the emission spectrum characteristic of the bioluminescent oxidation of luciferin. Thus the oxyluciferin-luciferase complex is the light-emitter. The binding is equimolar, with dissociation constant* K_D *equal to* 3×10^{-7} *mole per liter. The molecular weight of the luciferase, according to three different methods, is between 52,000 and 57,000; molecular activities of luciferase for the bioluminescence reaction and for the hydrolysis of oxyluciferin are 1600 and 2 per minute, respectively.*

The degradative pathway of the bioluminescent oxidation of *Cypridina* luciferin catalyzed by luciferase (Fig. 1) is now well established (*1, 2*). After a study by McCapra and Chang on the chemiluminescence of a model compound (*3*), Goto *et al.* made further studies on both model and natural compounds (*4, 5*) and concluded, on the basis of spectrographic data, that oxyluciferin is the light-emitting species in the chemiluminescence of luciferin in organic solvents. Although Goto *et al.* did not include data on the fluorescence of oxyluciferin in aqueous solution with luciferase, they alluded to the possibility that, when bound to luciferase, oxyluciferin could be, in effect, in a hydrophobic environment (*6*) and, in this circumstance, the actual light-emitter in the bioluminescence reaction. Our study demonstrates that the oxyluciferin-luciferase complex is the light-emitter and provides some new data concerning the properties of very highly purified luciferase.

Freshly collected specimens of *Cypridina hilgendorfii* were extracted first with cold methanol to remove the luciferin, and then with cold water, to remove luciferase. The aqueous extract was chromatographed six successive times, alternately on columns of DEAE-cellulose and Sephadex G-100; the active fractions were eluted from the DEAE-cellulose column by gradient concentrations of NaCl (*7*), and from the Sephadex G-100 column by a mixture of 0.01*M* sodium phosphate and 0.2*M* NaCl at *p*H 6.8. The material from the sixth column revealed a single band by disc electrophoresis at *p*H 9.5. Analytical ultracentrifugation resulted in a highly symmetrical peak in contrast to the slightly distorted peak found previously (*7*). The absorbance of a 1 percent solution (1-cm cell) at 280 nm was 9.63. Concentrated solutions were slightly pink, the wavelength of maximum absorbance being 525 nm, and the absorbance of a 1 percent solution (1-cm cell) being 0.2; however the color was irreversibly destroyed by sodium hydrosulfite without affecting the catalytic activity in bioluminescence. Fluorescence was that of a simple protein, with no peak above 345 nm. The sedimentation constant $s_{20,w}$ extrapolated to zero concentration was 4.66*S*, a value in satisfactory agreement with the 4.58*S* reported by Tsuji and Sowinski (*8*). By averaging these *S* values, using the previously reported diffusion constant of 7.9×10^{-7} cm²/sec (*7*), and assuming 0.73 as the partial specific volume, we calculated the molecular weight of luciferase to be 52,000, with a frictional ratio (f/f_0) of 1.09. Data from sedimentation equilibrium at 8750 rev/min indicated a molecular weight of 55,000 and gel filtration on a Sephadex G-100 column, calibrated by bovine serum albumin and by egg albumin, indicated a molecular weight of 57,000. These values are in fair agreement with each other, and with our reported values of 48,500 and 53,000 (*7*), but they are considerably lower than the 80,000 molecular weight reported by Tsuji and Sowinski (*8*). For present purposes 53,000 was taken as the molecular weight. The molecular activity (number of molecules of substrate transformed per minute per molecule of enzyme) for the luminescence reaction was found to be 1600 per minute when measured by a photomultiplier apparatus at 25°C; the reaction mixture contained $10^{-8}M$ luciferase and $10^{-5}M$ luciferin in a mixture of 0.05*M* sodium phosphate and 0.1*M* NaCl at *p*H 7.4. The hydrolase activity for the formation of etioluciferin from oxyluciferin was low; the molecular activity was 2 per minute when measured by decrease in absorption at 353 nm in the same buffer at 25°C and with $10^{-6}M$ luciferase and $10^{-4}M$ oxyluciferin (*9*).

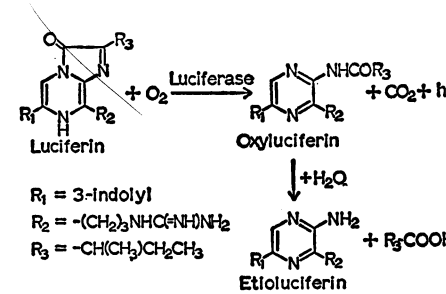

Fig. 1. Degradation of luciferin by luciferase.

Luciferin, oxyluciferin, and etioluciferin are each fluorescent in aqueous solution, but with different maximums (Table 1). The fluorescence of luciferin is fairly strong and does not change on addition of luciferase or when dissolved in butanol instead of water. However, the fluorescence of oxyluciferin or of etioluciferin is very weak in water, but becomes much more intense in an organic solvent or on addition of luciferase to the aqueous solvent. Moreover, with either substance the peak shifts to a shorter wavelength. In buffer, oxyluciferin bound to luciferase gave a fluorescence spectrum corresponding exactly to the emission spectrum of the bioluminescent reaction with the maximum at 465 nm (Fig. 2). No other compound or complex which could exist in this bioluminescence system gives a similar peak emission (Table 1).

The great increases in fluorescence intensity of oxyluciferin and etioluciferin (but not luciferin) in the presence of luciferase (Table 1) made it possible to do fluorometric titrations of lucifer-

Table 1. Fluorescence emission maximums (nanometers) and intensities (in arbitrary units shown in parentheses) for luciferin and reaction products, at $2 \times 10^{-6}M$ at 25°C, measured, at the indicated wavelength, with an Aminco-Bowman spectrophotofluorometer.

Solvent	Luciferin 430 nm (nm)	Oxyluciferin 360 nm (nm)	Etioluciferin 360 nm (nm)
0.05*M* Sodium phosphate – 0.1*M* NaCl, *p*H 7.4	535 (17)	510 (1)	503 (5)
n-Butanol	535 (18)	477 (103)	470 (180)
0.05*M* Sodium phosphate— 0.1*M* NaCl, *p*H 7.4 with $6 \times 10^{-6}M$ luciferase	535 (18)*	465 (100)†	480 (65)

* In the presence of sodium hydrosulfite to stop the luminescence reaction. (Visual observation indicated similar results with a vacuum.) † The solvent, containing luciferase ($4 \times 10^{-6}M$), was chilled in a cuvette to 0°C, and measurement was made within 10 seconds after oxyluciferin was mixed in, to minimize error due to hydrolysis to etioluciferin.

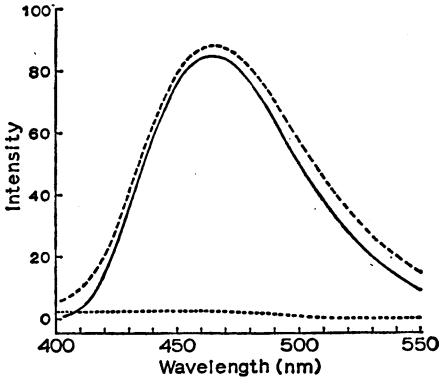

Fig. 2. Luminescence spectrum of luciferin plus luciferase (solid line) and fluoresence spectrum for the equimolar ($3 \times 10^{-6}M$) mixture of oxyluciferin and luciferase on excitation at 360 nm (broken line) in a mixture of 0.05*M* sodium phosphate and 0.1*M* NaCl, *p*H 7.4 at about 0°C within 15 seconds of the mixing. In reference to the latter spectrum, fluorescence without oxyluciferin (dotted line) and without luciferase (not shown) were both negligibly low.

ase with these substances. The data (Fig. 3) indicated that 1 mole of oxyluciferin binds hydrophobically per mole of luciferase, and, assuming that the increase in fluorescence is proportional to the amount of the oxyluciferin–luciferase complex so formed, we calculated the dissociation constant K_D to be $3 \times 10^{-7}M$ at about 0°C. The same result was obtained from titration data in terms of the amount of quenching of protein fluorescence taking place at 345 nm after being excited at 280 nm. Titration of luciferase with etioluciferin showed that the binding is equimolar, and that the calculated dissociation con-

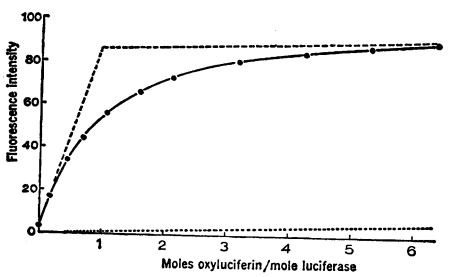

Fig. 3. Fluorometric titration of $2 \times 10^{-6}M$ luciferase with $10^{-4}M$ oxyluciferin, in a mixture of 0.05*M* sodium phosphate and 0.1*M* NaCl, *p*H 7.4. To minimize error due to hydrolysis, various amounts of oxyluciferin were quickly added to 1-ml portions of luciferase solution at 0°C, and the fluorescence measurements were done within 10 seconds of the additions. The broken straight lines are tangent to the titration curve at zero and at large excess concentrations of oxyluciferin, respectively. The dotted line shows the result when luciferase was omitted.

stant, $K_D = 2 \times 10^{-6}M$ at 25°C, was larger than that of oxyluciferin by nearly an order of magnitude. Attempts to obtain a value for the K_D of the luciferin-luciferase complex by methods of equilibrium dialysis or with Sephadex G-25 were unsuccessful because of the extreme instability of luciferin and the very small value to be expected for K_D which should be considerably smaller than the Michaelis constant of $5 \times 10^{-7}M$ at 25°C (*7*).

The foregoing evidence strongly supports our conclusion that the equimolar complex of oxyluciferin with luciferase in an excited state is the light-emitter of *Cypridina* bioluminescence. Moreover, the presence of only one hydrophobic binding center for oxyluciferin on the luciferase molecule suggests that the catalytic center for the light-emitting reaction of luciferin is also only one, and that these centers, if not identical, must be very close together.

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References and Notes

1. Y. Kishi, T. Goto, Y. Hirata, O. Shimomura, F. H. Johnson, in *Bioluminescence in Progress*, F. H. Johnson and Y. Haneda, Eds. (Princeton Univ. Press, Princeton, N.J., 1966), p. 89; *Tetrahedron Lett.* **1966**, 3427 (1966).
2. H. Stone, *Biochem. Biophys. Res. Commun.* **31**, 386 (1968).
3. F. McCapra and Y. C. Chang, *Chem. Commun.* **1967**, 1011 (1967).
4. T. Goto, S. Inoue, S. Sugiura, *Tetrahedron Lett.* **1968**, 3873 (1968).
5. T. Goto, S. Inoue, S. Sugiura, K. Nishikawa, M. Isobe, Y. Abe, *ibid.*, p. 4035.
6. L. Stryer, *Science* **162**, 526 (1968); G. M. Edelman and W. O. McClure, *Accounts Chem. Res.* **1**, 65 (1968).
7. O. Shimomura, F. H. Johnson, Y. Saiga, *J. Cell. Comp. Physiol.* **58**, 113 (1961).
8. F. I. Tsuji and R. Sowinski, *ibid.*, p. 125.
9. This hydrolase activity conceivably could indicate the presence of a contaminating enzyme, undetectable by ordinary methods, in the luciferase preparation. It could also indicate a property of the luciferase itself. While available evidence is insufficient to distinguish positively between the two alternatives, the ratio of luminescence activity to hydrolase activity was constant in the various fractions of the final chromatography, thus favoring the view that the hydrolase activity is indeed attributable to luciferase. In any event, the hydrolase activity can be eliminated as a significant factor in the luminescence reaction merely by using appropriate concentrations of luciferase and luciferin. Thus it has no essential role in the luminescence reaction, and any significant relation of this hydrolase activity to that of firefly luciferase is rather obscure [M. DeLuca and W. D. McElroy, *Biochem. Biophys. Res. Commun.* **18**, 836 (1965)].
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Centrioles of a Human Cancer: Intercellular Order and Intracellular Disorder

Abstract. *A continuous, symmetric, and periodic pattern of long-range spatial order has been observed among the centrioles of both normal and neoplastic cells. Consecutive ultrathin sections of lethal human esophageal cancers have revealed a defect in the centriolar angle with random angularity and random, long, intercentriolar distances.*

One of the most interesting revelations of electron microscopy has been that ubiquitous constancy with which gross macromolecular aggregates comprise a geometrical pattern of fine structure by which centrioles can be readily identified (*1*). Furthermore, at least among nondividing somatic animal cells, the two centrioles of a single cell are close to each other and are regularly disposed so that their long axes form a right angle, a conspicuous exception to the free-form curvatures that characterize these dimensions of biology (*2*). Heretofore unasked is the question of whether the constant geometry of centriolar ultrastructure and the constant orthogonal relationship of the two centrioles of a single cell is part of a larger intercellular pattern of spatial order among these tiny organelles. This can now be studied, for a procedure has been perfected which consistently yields information from an unlimited number of consecutive sections (700 Å thick) of intact tissues prepared for ultramicrotomy and electron microscopy by conventional methods (*3*).

To minimize the effect of complex time-variable functions upon the spatial orientation of centrioles, tissues were fixed *in situ* or promptly after atraumatic excision, and sections were cut consecutively from the tissue-fixative interface. The area selected for study was centered in a precisely cut block face (0.14 by 0.28 mm) oriented normal to the knife edge. One thousand consecutive serial sections (700 Å thick) were then cut as straight ribbons, none of which exceeded a length of eight sections, each consecutive ribbon being mounted in exact alignment with an unobstructed slot (0.12 by 2.5 mm) in a special specimen grid. Each mounted ribbon was then permanently inserted into a separate electron microscope specimen holder, after which it was checked by electron microscopy