Protein–Protein Interactions in the *Pholas dactylus* System of Bioluminescence†

J. P. Henry* and C. Monny

ABSTRACT: *Pholas* luciferin is a glycoprotein possessing a prosthetic group with an absorption at 307 nm (ϵ = 11 800). Oxyluciferin lacks this chromophore but has a weak absorption band at 355–360 nm. Both luciferin and oxyluciferin have molecular weights of 34 000 and show similar amino acid compositions and sugar and sulfate contents. The two proteins have very similar electrophoretic properties when native but can be separated under denaturing conditions. No significant fragmentation of luciferin occurs under these conditions. This change in electrophoretic mobility is seen when oxyluciferin is prepared by the action of luciferase and also, at least partially, when luciferin is oxidized nonenzymatically. Two molecules of luciferin can be oxidized by one molecule of luciferase with emission of light in the absence of spectral modification at 307 nm. The change in absorption at 307 nm thus occurs after light emission, and it is possible that this modification (as well as the electrophoretic changes) is a secondary event accompanying dissociation of oxidized luciferin from the enzyme–substrate complex. Luciferase and luciferin or oxyluciferin (labeled with ^{125}I) form complexes with a stoichiometry of two molecules of luciferin or oxyluciferin per molecule of enzyme, both sites on the enzyme having the same affinity. The association between oxyluciferin and luciferase is an entropy-controlled process ($\Delta H > 0$, $\Delta S > 0$) with $K_d = 1.7 \times 10^{-8}$ M. The small value of the dissociation constant is a result of rapid association with $k_a = 1.6 \times 10^7$ M^{-1} s^{-1}. The luciferin–luciferase complex has been studied under conditions which block the catalytic reaction (acid pH and 0 °C). Under these conditions, whereas the rate constants of association of oxyluciferin or luciferin with luciferase are quite similar, the dissociation rate constants differ widely; indeed, dissociation of luciferin from the luciferin–luciferase complex is too slow to be measured. The equilibrium dissociation constant under these conditions is estimated to be 10^{-11} M.

The bioluminescent system from the boring mollusc *Pholas dactylus* is unique in that both luciferase and luciferin are glycoproteins. The presence of a prosthetic group in the luciferin molecule has been indicated by spectrophotometric techniques (Henry et al., 1970, 1973, 1975). It has also been shown that *Pholas* luciferin emits light when treated with a striking variety of reagents in the absence of luciferase, and that superoxide ion $O_2^{-}\cdot$ is a reactive intermediate common to many of the light-emitting reactions, enzymatic as well as nonenzymatc (Henry and Michelson, 1970, 1973; Henry et al., 1973, Michelson and Isambert, 1973). Properties of the luciferase molecule have recently been described (Henry et al., 1975). It is an acidic molecule, very asymmetric, which contains cupric copper as part of the active site. It is isolated either as a dimer with a molecular weight of 310 000 (A form) or as a complex of this dimer with an inactive modification of luciferin (B form). The B form of the enzyme is the major species under the described purification procedure.

Protein–protein interactions play an important role in the luciferin–luciferase reaction, since they control product as well as substrate associations with the enzyme. In the present communication, we describe characteristics of luciferin and oxyluciferin and their complexes with luciferase.

Experimental Section

Apparatus for Measurement of Light Emission. Light emission was characterized by maximal intensity (I_{max}) and by total light emitted (L), which were measured simultaneously with the instrument previously described (Henry et al., 1970; Henry and Michelson, 1973). The apparatus was calibrated by the technique of Lee (Lee et al., 1968) and by that of Hastings and Weber (1963). As the two methods gave results which differ by a factor of 3, the results have been expressed in arbitrary units, 1 light unit being equal to 6.1×10^8 photons/mL according to Lee et al. or to 20.7×10^8 photons/mL according to Hastings and Weber.

Purification of Pholad Luciferin. Luciferin was routinely assayed by the total light (L) emitted after addition of 1 mL of a degassed solution of ferrous sulfate (0.3 mM) to the protein sample suspended in 2 mL of 0.15 M phosphate buffer (pH 7.0) containing 0.75 M NaCl (Henry and Michelson, 1973).

The purification technique used was that of Henry (Henry et al., 1970) with the following modifications. The crude luciferin solution was obtained by homogenization of the acetone powder (4 g) from dissected luminous organs with an aqueous solution (30 mL) of diethyl dithiocarbamate (1 mM) in a Polytron grinder (Kinematica GMBH, Lucerne) operating at full speed for 60 s. The extract was centrifuged for 15 min at 28 000*g* in a refrigerated Sorvall centrifuge. The supernatant (crude luciferin) was then purified as already described (acidic precipitation, ammonium sulfate precipitation and Sephadex G-100 filtration) but chromatography on DEAE[1]-Sephadex (A-50) was added as a final step. The active material (25 mg) from the Sephadex G-100 column was adjusted to 0.1 M NaCl and 0.05 M phosphate (pH 7.0) and poured onto a column (1.5 × 23 cm) of DEAE-Sephadex which had been equilibrated

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[1] Abbreviations used are: DEAE, diethylaminoethyl; Tris-HCl, 2-amino-2-hydroxymethyl-1,3-propanediol hydrochloride; K_d, equilibrium dissociation constant; ΔH_a, ΔF_a, and ΔS_a, enthalpy, free energy, and entropy changes of the association reaction, respectively; k_a and k_d, association and dissociation rate constants.

with the same buffer. The column was washed with 40 mL of buffer and then a gradient of NaCl (from 0.1 to 0.8 M) was applied. The active fractions were pooled, concentrated with Diaflo PM-10 membranes, dialyzed against 0.01 M phosphate (pH 7.0) buffer, and kept frozen at −20 °C. Alternatively, the DEAE-Sephadex column was charged at pH 4.8 in 0.05 M acetate buffer containing 0.1 M NaCl and eluted with a gradient of NaCl (from 0.1 to 0.6 M).

Purification of Pholad Oxyluciferin. The purification was followed by disc gel electrophoresis (Davis, 1964). Acetone powder (1 g) of luminous organs was dispersed in 30 mL of 0.01 M phosphate buffer (pH 7.0) with a Polytron grinder operating at full speed for 50 s. The homogenate was centrifuged at 28 000*g* for 15 min. The supernatant was then kept at room temperature until more than 95% of the luciferin activity had disappeared. This extract was used as a source of crude oxyluciferin and purified by the technique previously described for luciferin purification. Alternatively, oxyluciferin was obtained during preparation of luciferase, after chromatography on DEAE-Sephadex of the acid extract (Henry et al., 1975).

Purification of Pholad Luciferase. The complexed form of luciferase (B form) was purified by the technique previously described (Henry et al., 1975). The free form (A form) of the enzyme was obtained by purification of the first peak of activity obtained by chromatography on DEAE-Sephadex, using the same technique (ammonium sulfate precipitation and centrifugation in a cesium chloride gradient). The absence of inactive luciferin in this preparation was checked by disc gel electrophoresis in sodium dodecyl sulfate.

Protein Determinations. The protein content of impure preparations was determined by the biuret reaction using crystalline bovine serum albumin as a standard or by measuring the absorbance at 280 and 260 nm (Layne, 1957). For purified samples, the concentration was derived from the absorbance assuming a specific absorbance $E_{280nm}^{1\%,1cm}$ of 12.7 and 14.9 for oxyluciferin and luciferin, respectively. These values were obtained from measurements of the refractive index of dialyzed solutions with an interferometer (Zeiss Jena, L 13 model) calibrated with trypsin, α-chymotrypsin, bovine serum albumin, pancreatic ribonuclease, D-(+)-glucose, D-(+)-mannose, L-(−)-fucose, and using a sugar content of 20% for the two proteins.

Molecular Weight Determinations. Ultracentrifugal Studies. Sedimentation equilibrium studies were performed with a Beckman Spinco analytical ultracentrifuge. Interference optics were used and the double-sector cell was filled to a column height of 3 mm. The experimental conditions maintained protein throughout the solution and gave a small but finite concentration of protein at the meniscus at equilibrium. The buffer used was 0.05 M Tris-HCl (pH 7.5) in 0.1 M NaCl. The results were plotted as log of the protein concentration against the square of the distance from the center of rotation. The line obtained was the least-squares fit of experimental points. The proteins were studied at several concentrations and at several rotor speeds. In the calculation of molecular weights, a partial specific volume of 0.707 was assumed. This value was derived from the chemical composition of luciferase.

Determination by Gel Filtration and Density Gradient Centrifugation (Siegel and Monty, 1966). Stokes radii were determined by filtration on Sephadex G-200 in the buffer described for ultracentrifugal studies. Dextran Blue and tritiated water were used to characterize the column (1.15 × 53.5 cm). Calibration was done with the following markers which were assayed by their enzymatic activity or their absorbance: cytochrome *c* (r = 17.4 Å), horse myoglobin (r = 10 Å), human erythrocuprein (r = 24.6 Å), ovalbumin (r = 27.7 Å), horse liver alcohol dehydrogenase (r = 35 Å), and bovine serum albumin (r = 36.1 Å). The Stokes radii were calculated according to Ackers (Ackers, 1967).

Sedimentation coefficients were determined by centrifugation in sucrose gradients (Martin and Ames, 1961). Samples in 0.1 M NaCl-0.05 M Tris-HCl were centrifuged for 20 h at 50 000 rpm in the SW 65 rotor. Three determinations were made with the following markers: horse liver alcohol dehydrogenase (5.1 S), human erythrocuprein (3.3 S), horse myoglobin (2.0 S), and ovalbumin (3.55 S).

Gel Electrophoresis. For disc gel electrophoresis, the technique of Davis (Davis, 1964) was followed. The proteins were stained with Coomassie Brilliant Blue and the sugars with Schiff's reagent (Henry et al., 1965). When the mobility was studied as a function of acrylamide concentration, the ratio acrylamide–bisacrylamide (w/w) was kept constant and equal to 30 (Hedrick and Smith, 1968). The same technique was used with 8 M urea present in the gel but then the samples were prepared in the following way: to the solution (65 μL) containing the mixture of proteins (300 μg) solid urea (50 mg) and β-mercaptoethanol (5 μL) were added and the mixture was heated at 100 °C for 2 min. After addition of 5 μL of 0.01% bromophenol blue, aliquots (10 μL) were layered on gels.

Sodium dodecyl sulfate–polyacrylamide gels were carried out according to Weber and Osborn (1969) or to Laemli (1970). To calibrate the gels, the following markers were used: trypsin (molecular weight 23 000), yeast alcohol dehydrogenase (molecular weight 37 000), bovine serum albumin (molecular weight 67 000 and 134 000).

Test for Protease Activity of Pholas Luciferase Preparations. To purified luciferase (30 μg) in 0.2 M Tris-HCl buffer (pH 7.5) (0.1 mL), [^{125}I]casein (80 μg) was added. The mixture was incubated for 120 min at 37 °C and then the reaction was stopped by addition of 30 μL of trichloroacetic acid (50%). The precipitate was centrifuged and the radioactivity of the supernatant measured in 5 mL of Bray's scintillation liquid.

Amino Acid Analysis. Analyses were performed on protein samples hydrolyzed for 16 h at 110 °C with 6 N HCl in tubes sealed in vacuo, using a Unichrom amino acid analyzer. Cysteine was determined as cysteic acid after oxidation with performic acid. The tryptophan content was obtained by the spectrophotometric method of Edelhoch (1967).

Sugar Analysis. Sugars were analyzed by gas–liquid chromatography of the trimethylsilyl derivatives of the *O*-methyl glucosides obtained by methanolysis of the protein (Chambers and Clamp, 1971). They were identified by comparison with known markers and estimated quantitatively using a standard of mannitol. Glucosamine was also assayed by the ninhydrin reaction on the Unichrom analyzer after a 4-h hydrolysis at 100 °C with 3 N HCl.

Sulfate and Phosphate Analysis. Organic phosphate contents were analyzed by the technique of Ames (1966). Organic sulfate analyses were done according to Terho and Hartiala (1971) modified in the following way. The samples were hydrolyzed for 90 min with 1 N HCl, at 100 °C, in sealed tubes. The tubes were then carefully dried overnight in an oven before addition of the reagents. All volumes were divided by a factor of 5.

Preparation of ^{125}I-Labeled Oxyluciferin (Marchalonis, 1969). To purified oxyluciferin (250 μg) in 0.05 M phosphate buffer (pH 7.0) were added lactoperoxidase (1.25 μg) and Na^{125}I (1 mCi) in a final volume of 50 μL. The reaction was initiated by addition of 1 μL of H_2O_2 (8.8 mM) and the tube

was gently shaken at room temperature. After 30 min, phosphate buffer (0.5 mL) was added and the solution was then extensively dialyzed with multiple buffer changes.

The radioactivity was measured in 5 mL of NE 250 liquid scintillator (Nuclear Enterprises) using the ^{14}C channel of an Intertechnique scintillation spectrometer. More than 50% of the radioactivity was incorporated in the protein, giving a specific activity of 3.2 mCi/mg of protein. It was calculated that only 5% of the protein molecules were iodinated. Ascending chromatography on Whatman paper No. 1 in 1-butanol–acetic acid–water (4:1:1) showed that more than 98% of the radioactivity was attached to the protein.

Dissociation Constants of the [^{125}I]Oxyluciferin–Luciferase Complex. All experiments were carried out with purified free luciferase (A form) and freshly dialyzed [^{125}I]oxyluciferin. Only plastic tubes or glassware coated with Repelcote (Hopkin and Williams) were used. Mixtures of luciferase and oxyluciferin (75 μL) containing 3 to 20 times excess of luciferase were incubated for 60 min at the experimental temperature. They were layered onto a column (0.6 × 20 cm) of Sephadex G-200 equilibrated with the same concentration of [^{125}I]oxyluciferin in 0.05 M Tris-HCl buffer (pH 7.5) by passing 2 void volumes of oxyluciferin solution. Fractions (200 μL) were collected and weighed and their radioactivity was measured in 5 mL of NE-250 liquid scintillator (Figure 3). The amount of complex formed was calculated assuming that the baseline represented free ligand and that the area between the first peak and the baseline was due to complex formation. Areas of the first (positive) and of the second (negative) peaks were very similar.

To estimate the enthalpy of the association process, the dissociation constant was measured at 5, 15, 25, and 35 °C using a thermostated Sephadex G-200 column. The results were plotted according to Van't Hoff.

Dissociation Kinetics of the Luciferin–Luciferase Complex. Purified luciferase (39.4 pmol) was incubated with luciferin (113.6 pmol) in 4 mL of 0.05 M acetate buffer (pH 4.8) at 0 °C for 5 min. Oxyluciferin (56.8 nmol) was then added and aliquots (0.5 mL) were withdrawn at various times and assayed for luciferin–luciferase complex by transferring them into 2.5 mL of 0.1 M Tris-HCl buffer (pH 8.5) containing 0.5 M NaCl. An incubation mixture without oxyluciferin was run as control.

Ratio of Equilibrium Dissociation Constants of Luciferin– and Oxyluciferin–Luciferase Complexes. Purified free luciferase (9.6 nM) and oxyluciferin (2.8 μM) were incubated at 0 °C for 5 min in 5 mL of 0.05 M acetate buffer (pH 4.8). Luciferin (31.2 nM) was then added (zero time) and the evolution of the mixture was followed by transferring 0.5-mL aliquots to 2.5 mL of 0.05 M Tris-HCl buffer (pH 8.5). From the flash height, the number of luciferase sites combined with a luciferin molecule was calculated. Another aliquot (0.1 mL) was assayed by Fe^{2+}-PO_4^{3-} to give the luciferin activity remaining in the incubation mixture. The level of luciferin activity was kept constant by addition of fresh luciferin. Equilibrium was obtained after 8 h. The ratio $K_d[I_{ox}]/K_d[I]$ was calculated assuming that free luciferase, luciferase–luciferin (1:1), and luciferase–oxyluciferin (1:1) concentrations were negligible and that the two sites of the enzyme were equivalent and independent.

Results

Characteristics of Luciferin and Oxyluciferin. Since luciferase was strongly inhibited by diethyl dithiocarbamate (Henry et al., 1975), we have modified the earlier purification procedure of luciferin (Henry et al., 1970) by using this inhibitor during the preparation. The step involving thermal denaturation of luciferase, which also destroyed a large part of the luciferin activity, was eliminated. Under these conditions, the purified luciferin was about ten times more active than the earlier preparations which, although homogeneous with respect to the protein, were 90% inactive. The quantum yield of this luciferin was 0.09 if calibrated by the luminol method of Lee (Lee et al., 1968) or 0.27 if calibrated according to Hastings (Hastings and Weber, 1963). Oxyluciferin was purified by the same procedure as for luciferin, except that diethyl dithiocarbamate was omitted.

Protein concentrations were measured by interferometry using various proteins and sugars to calibrate the apparatus and using a sugar content of 20% for luciferin and oxyluciferin. The values of $E_{280\,nm}^{1\%,1cm}$ thus determined were 14.9 for luciferin and 12.7 for oxyluciferin.

The Stokes radii obtained by filtration on Sephadex G-200 according to Ackers (1967) were identical for luciferin and oxyluciferin, with a value of 29.8 Å. From this value, the diffusion constant was calculated to be 7.2×10^{-7} cm^2 s^{-1}. Sedimentation constants determined by centrifugation in sucrose gradients also gave identical values of 3.05 S for both luciferin and oxyluciferin. Molecular weights determined from sedimentation constants and Stokes radii were estimated to be 35 000, assuming a partial specific volume of 0.707, the figure used for luciferase (Henry et al., 1975). Luciferin is quite asymmetric (f/f_0 = 1.4) but considerably less so than luciferase (f/f_0 = 1.8). Ultracentrifugation approaching equilibrium gave somewhat scattered values as a function of concentration for oxyluciferin. The average value was found to be 34 600 ± 5400 (p = 0.1, n = 4, Student's t test). This dispersion could well be due to different degrees of association of the molecule, since after heating of the luciferin for 5 min to 65 °C a dimer was formed with a molecular weight of 66 500 (value calculated by extrapolation to zero concentration). In all further work, a molecular weight of 34 000 for luciferin and oxyluciferin was used. Dissociation of the dimer could be achieved in presence of sodium dodecyl sulfate and β-mercaptoethanol.

Under nondenaturing conditions, luciferin and oxyluciferin were not separated by disc electrophoresis on polyacrylamide gels, and with concentrations of acrylamide lower than 8% both molecules migrated with the front. This behavior may be due to their very low isoelectric point (p$I \leq 3.5$). At concentrations of acrylamide greater than 8%, a slight indication of separation was observed. However, under conditions of denaturation in 8 M urea (after heating with β-mercaptoethanol) luciferin and oxyluciferin were separated by electrophoresis on gels containing from 4 to 11% acrylamide. The two molecules had the same molecular weight and differed in charge, since in each case the logarithm of the mobility was a linear function of the concentration of acrylamide and the two lines were parallel (Hedrick and Smith, 1968). Since the molecular weights of luciferin and oxyluciferin are identical, no difference in mobilities in sodium dodecyl sulfate gels would be expected. In fact, a significant difference was observed reflecting the fact that with glycoproteins the mobility is not uniquely a function of molecular weight (Segrest and Jackson, 1972). Thus, apparent molecular weights determined by electrophoresis on sodium dodecyl sulfate gels calibrated with suitable markers varied with the concentration of acrylamide used in the gel (Table I). The difference between the electrophoretic properties of luciferin and oxyluciferin in sodium dodecyl sulfate gels was a result of luciferase action, since when complete

TABLE I: Electrophoretic Mobilities of Luciferin and Oxyluciferin on Sodium Dodecyl Sulfate-Polyacrylamide Gels.[a]

Acrylamide concn (%)	Mobility (R_m)		Apparent mol wt	
	I	I_{ox}	I	I_{ox}
5	0.70	0.76	48 000	40 000
7.5	0.51	0.62	44 000	33 000
10	0.44	0.48	36 000	32 000
12.5	0.32	0.37	32 800	29 500

[a] The electrophoresis and the apparent molecular weight determinations were done according to Weber and Osborn (1969). The markers used are given under Experimental Section.

TABLE II: Enzymatic Conversion of Luciferin to Oxyluciferin.[a]

	Mobilities (R_m)[b]	
	System A	System B
Luciferin	0.57-0.55	0.78-0.78
Oxyluciferin	0.62-0.62	0.83-0.82
Luciferin incubated with luciferase	0.62-0.61	0.82-0.82

[a] Luciferin (0.18 mg) and luciferase (0.13 mg) were incubated in 0.4 mL of 0.1 M Tris-HCl buffer (pH 8.5) containing 0.5 M NaCl and 0.5 mg/mL BSA for 4 h at room temperature. Ascorbate was added twice, at 10 μM final concentration. The reaction was followed by assaying luciferin activity on 2-μL aliquots. The controls were treated in the same way and it was shown that at the end of the incubation period there was no inactivation of the luciferin. The samples were denatured by boiling them for 2 min in presence of 2% sodium dodecyl sulfate and 1% β-mercaptoethanol. They were dialyzed against electrophoresis buffers. [b] Gel systems A and B were those of Weber (1969) and Laemli (1970), respectively.

TABLE III: Chemical Analyses of *Pholas* Luciferin, Oxyluciferin, and Luciferase.[a]

Component	Luciferin		Oxyluciferin		Luciferase	
	No. of residues	% of total wt	No. of residues	% of total wt	No. of residues	% of total wt
Amino Acids						
Asp	31.2	10.6	31.3	10.6	338.3	12.6
Thr	20.0	5.9	18.2	5.4	175.8	5.7
Ser	7.1	1.8	7.6	1.9	167.0	4.7
Glu	25.0	9.5	25.9	9.8	257.7	10.7
Pro	5.3	1.5	7.2	2.1	109.0	3.4
Gly	13.2	2.2	13.7	2.3	257.1	4.7
Ala	9.8	2.0	10.2	2.1	150.9	3.5
Val	14.6	4.3	12.9	3.8	101.6	3.3
Met	8.3	3.2	10.4	4.0	76.9	3.3
Ile	9.0	3.0	8.2	2.7	130.2	4.8
Leu	9.9	3.3	9.1	3.0	173.1	6.3
Tyr	7.5	3.6	7.2	3.5	90.1	4.7
Phe	8.1	3.5	7.2	3.1	90.5	4.3
His	0	0	0.3	0.1	53.4	2.4
Lys	7.8	2.9	4.9	1.8	127.0	5.3
Arg	8.6	4.0	7.9	3.61	152.1	7.7
Cys	8.5	2.6	6.8	2.1	61.6	2.0
Trp	6.2	3.4	6.2	3.4	56.5	3.4
Sugars						
GlcN	11.1	6.6	11.3	6.8	39.4	2.6
Fuc	9.8	4.2	10.6	4.6	86.4	4.1
Man	7.1	3.4	6.6	3.1	57.0	3.0
Gal	5.2	2.5	5.1	2.4	20.9	1.1
SO_4^{2-}	2.6	0.7	3.3	0.9	*b*	

[a] The results were calculated assuming molecular weights of 34 000 for luciferin and oxyluciferin and of 310 000 for luciferase (A form). The chemical composition of luciferase (A form) was obtained by subtracting one molecule of luciferin from luciferase (B form) composition (Henry et al., 1975). [b] Not investigated.

enzymatic oxidation of the luciferin was achieved, the product of the reaction had the mobility of oxyluciferin (Table II). Conversion to the faster moving band (oxyluciferin) did not occur on heating luciferin (5 min at 65 °C), and the product thus obtained, which had no luciferin activity, was not transformed by luciferase. Likewise, the transformation as well as the emission of light was completely inhibited by addition of diethyl dithiocarbamate to the luciferin-luciferase mixture. Conversion of luciferin could also be observed, at least partially, when oxidation was performed with peroxidase bound to a solid support or with O_2^-· generated by the system $Fe^{2+}/O_2/PO_4^{3-}$. The incomplete transformation found in both cases (data not shown) might be the result of a dark reaction, since the quantum yield was not measured. Thus, the variation in electrophoretic mobility was directly related to oxidation of luciferin by luciferase with emission of light rather than a residual proteolytic activity in the preparation. Indeed, no proteolytic activity could be detected when iodinated casein was used as a substrate.

The chemical compositions of luciferin and oxyluciferin are given in Table III. It can be seen that compositions in sugars are identical and the small differences observed in the amino acid composition may only reflect technical difficulties. The total sugar content represents 20% of the molecular mass. Both luciferin and oxyluciferin contain three sulfate residues, but no phosphate and iron were detected. The presence of sulfate residues explains the extremely low isoelectric point in luciferin and oxyluciferin. The sum of the components of the quantitative analyses accounts for about 85% of the determined molecular weight of luciferin and oxyluciferin. No estimate of the size of the prosthetic group is as yet available but it is unlikely that this alone could account for the remaining part of the molecule.

In confirmation of earlier work (Henry et al., 1973) but using more active luciferin preparations, we have shown that the transformation of luciferin to oxyluciferin is accompanied by the disappearance of a band at 307 nm and the appearance of bands at longer wavelengths. The difference spectrum at pH 8.5 in the presence of 3 μM ascorbate (Figure 1) showed positive bands at 255 and 360 nm and a negative band at 307 nm with a shoulder at 282 nm. This negative band has an ϵ of 11 800 at 307 nm. In the absence of ascorbate, the enzymatic reaction of luciferin-luciferase is extremely limited both with respect to light emission and appearance of these bands. Indeed, it could be estimated that under such conditions one molecule of enzyme oxidized only 2.5 molecules of luciferin. Addition of limiting quantities of ascorbate stimulated light emission and reduced optical density at 305 nm, the two phenomena being correlated (Figure 2). Measurement of absorption at 255 nm showed that ascorbate was consumed in the reaction, but the stoichiometry has not been determined.

Interaction between Luciferase and Oxyluciferin. In order to study this interaction, the oxyluciferin was labeled by iodination with ^{125}I using lactoperoxidase. The product obtained under optimal conditions had a specific activity of 3.2 mCi/mg

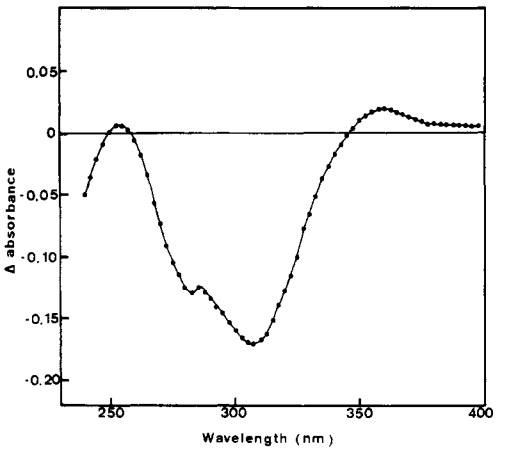

FIGURE 1: Changes in the absorption spectrum of luciferin (14.5 μM) during oxidation by luciferase (1.1 μM). The difference spectrum between a cuvette containing the mixture of luciferin and luciferase in 0.9 mL of 0.1 M Tris-HCl buffer, pH 8.5, and 0.5 M NaCl and two cuvettes (in the reference compartment) containing separated solutions of luciferin and luciferase at the same concentrations was recorded with a Cary 14 spectrometer. Four additions of ascorbate (3 μM) were made to the luciferin-luciferase cuvette. The spectrum was recorded after 120 min and corrected for variation of the baseline.

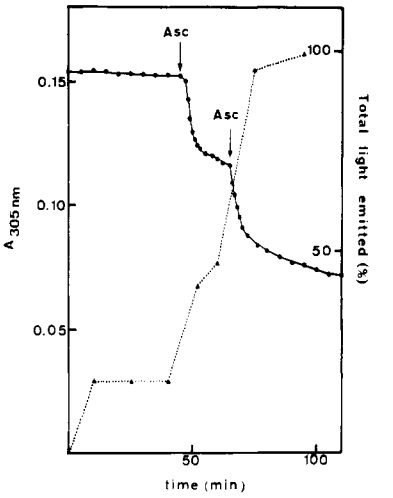

FIGURE 2: Correlation between light emission and absorption at 305 nm. Luciferin (7.3 μM) and luciferase (0.55 μM) were mixed in 0.8 mL of 0.067 M Tris-HCl, pH 8.5, containing 0.5 M NaCl. Two additions of ascorbate (4 μM) were made. Changes in absorption were measured in a Cary 14 spectrometer and the total light emitted was estimated from measurements of the activity of 3-μL aliquots assayed by $Fe^{2+}/O_2/PO_4^{3-}$.

of protein, but the overall yield indicated that only 5% of the molecules were labeled.

The experimental procedure used to demonstrate complex formation involved preincubation of the radioactive oxyluciferin with luciferase followed by gel filtration through Sephadex G-200. Since luciferase has a molecular weight of 310 000, all radioactivity found in the void volume is due to luciferase-oxyluciferin complex. Using this technique, it was found that 1.8 molecules of oxyluciferin were complexed with one molecule of luciferase under conditions where the total recovery of radioactivity applied to the column was 89%. Other experiments indicated somewhat lower values but the average of several experiments gave a figure of 1.64. It may be noted that, since the concentration of the complex under study was of the order of 100 K_d (see below), a correction should be applied to account for dissociation; this gives an average value of 1.8 molecules of oxyluciferin per molecule of luciferase. A second value was determined using the technique of Hummel and Dryer (1962; Fairclough and Frutton, 1966) in which the same column of Sephadex was equilibrated with radioactive oxyluciferin and a known quantity of luciferase was passed through (Figure 3). Again, the amount of radioactivity found in the void volume reflected oxyluciferin-luciferase complex formed, but since the concentrations of proteins used were of the order of K_d, complex formation was incomplete. For each experiment, the concentration of bound oxyluciferin was determined. Using different concentrations of oxyluciferin and luciferase, a number of values were determined, which were plotted in Scatchard form. Intersections with the abscissa gave values of 1.64 at 25 °C and 1.72 at 35 °C, with an average value of 1.68 molecules of oxyluciferin per molecule of luciferase, assuming that the K_d for iodinated oxyluciferin did not differ significantly from that of oxyluciferin. Determination of K_d from the experiments at 25 °C gave a figure of 1.72×10^{-8} M. Variation of K_d as a function of temperature gave a value of +3.49 kcal mol^{-1} for ΔH_a, whereas ΔF_a was determined to be −10.6 kcal mol^{-1} and ΔS_a +47.3 cal mol^{-1} deg^{-1}.

Association (k_a) and Dissociation (k_d) Rate Constants of Luciferase and Oxyluciferin. Mixtures of luciferase and oxyluciferin at sufficiently high concentrations were preincubated at pH 7.5 for 3 min. A fivefold excess of luciferin was then injected directly into the cuvette in the apparatus for measuring light emission. Light emission as a function of time was then followed and compared with a control in which no preincubation with oxyluciferin was performed. The difference between the two curves was plotted in a semilog form (Figure 4) to give a first-order constant with a value of 0.27 s^{-1}. This value was independent of the concentration of luciferase used in a series of experiments. The half-life derived from this curve is 2.6 s. From the value of K_d earlier determined (1.72×10^{-8} M) and since $K_d = k_d/k_a$, the rate constant of association was determined to be 1.57×10^7 M^{-1} s^{-1}.

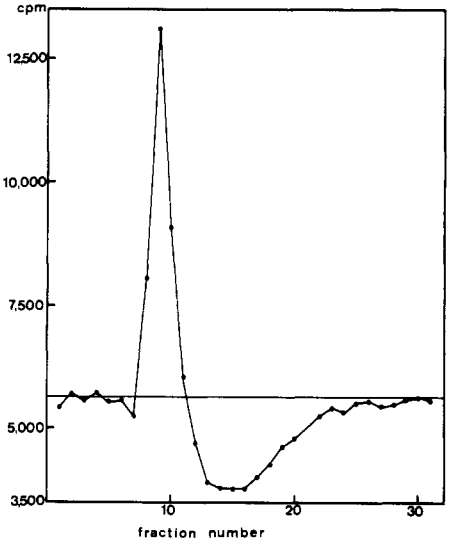

FIGURE 3: Binding of [^{125}I]oxyluciferin to luciferase (see Experimental Section). Filtration of a mixture (75 μL) of [^{125}I]oxyluciferin (0.17 pmol) and of luciferase (2.9 pmol) through a Sephadex G-200 column (0.6 × 20 cm) equilibrated with [^{125}I]oxyluciferin (2.25 nM).

Interaction between Luciferin and Luciferase. We have earlier shown (Henry et al., 1970) that luciferin forms a relatively stable complex with luciferase at acidic pH (or at neutral

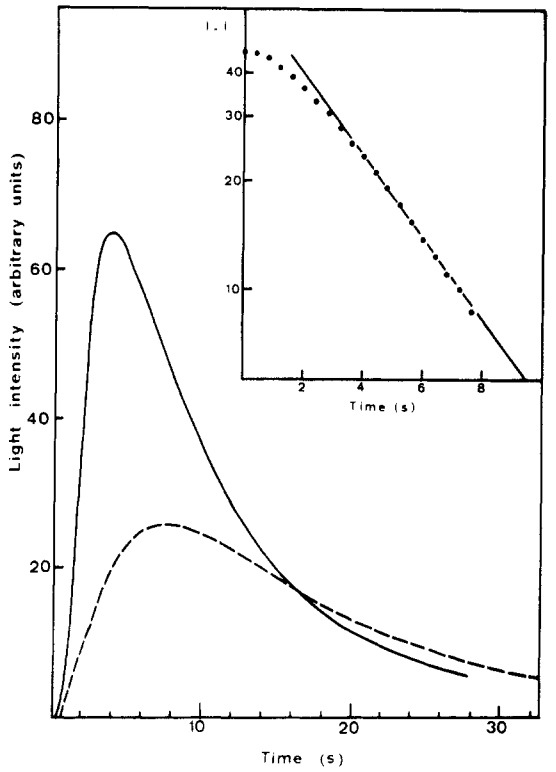

FIGURE 4: Dissociation of the oxyluciferin-luciferase complex. Oxyluciferin (0.15 μM) and luciferase (0.1 μM) were incubated for 3 min at room temperature in 0.5 mL of 0.05 M Tris-HCl buffer (pH 7.5). To this mixture maintained in front of the photomultiplier, luciferin (0.7 μM) was added with a Hamilton syringe and the light emission was recorded (dotted line). As a control, the same experiment was repeated without oxyluciferin (solid line). The decrease of the difference between the two curves was plotted in a semilog form as a function of time (Inset). $I - I_0$ was maximal at 3.2 s.

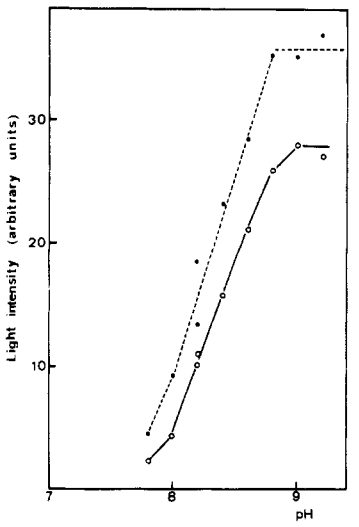

FIGURE 5: Effect of final pH on the assay of luciferin-luciferase complex. Luciferin (39 pmol) was incubated with either luciferase A (7 pmol) (●) or luciferase B (14 pmol) (○), in 0.5 mL of 0.05 M acetate buffer, pH 4.8, at 0 °C for 3 min. The mixture was then injected into 2.5 mL of 0.1 M Tris-HCl buffer containing 0.5 M NaCl. The light emission was recorded and the pH was measured after reaction.

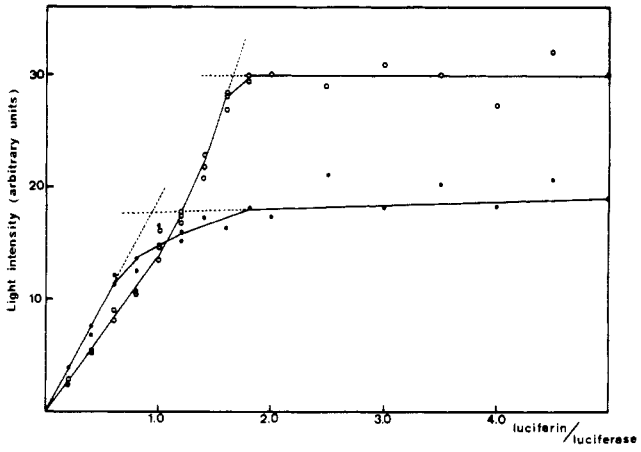

FIGURE 6: Titration of the B form of luciferase by luciferin. To B-luciferase (7.3 pmol) in 0.5 mL of 0.05 M acetate buffer (pH 4.8) various amounts of luciferin were added and incubated for either 5 (●) or 30 min (○) at 0 °C. The incubated solutions were then injected into 2.5 mL of 0.1 M Tris-HCl buffer (pH 9.0) containing 0.5 M NaCl and the light emission was measured.

pH under strictly anaerobic conditions). The decrease of activity which is observed is not a result of enzyme inactivation at acidic pH (Henry et al., 1975). A possible explanation is an incomplete freezing of the enzymatic reaction. At pH 4.8 in 0.05 M acetate buffer the complex luciferin-luciferase has a half-lifetime $\geq$ 120 min at 0 °C, whereas at 20 °C the half-life is about 45 min. This stability at pH 4.8 was used to study the interaction between luciferin and luciferase. The quantity of complex formed can be readily followed, since injection into buffer at alkaline pH (8.5-9) gives a flash of light which corresponds to the quantity of preformed complex. Final pH for activation of the complex with concomitant emission of light plays an important role, since both I_{max} and total light emitted (L) in 30 s increase notably with increase in pH (Figure 5). The characteristics of light emission after a pH jump of the preformed luciferin-luciferase complex to alkaline pH are somewhat different from those observed by the direct action of Fe^{2+}-PO_4^{3-} on luciferin (Henry and Michelson, 1970). Thus, I_{max} is about four times greater; that is, the kinetics of oxidation as indicated by light emission are much more rapid, whereas the quantum yield (total light emitted in 1 min) is lower and corresponds to about 50-70% of that of the nonenzymatic reaction.

Using this approach, luciferase was titrated by preincubation of a constant quantity of enzyme with varying amounts of luciferin at pH 4.8; aliquots were then injected into buffer at alkaline pH and the quantity of preformed complex was estimated from I_{max} as well as L. At pH 8.5, using 4.8 pmol of luciferase, the equivalence point or saturation was obtained at 13.75 pmol of luciferin, to give a ratio luciferin-luciferase of 2.86, whereas with ten times less luciferase the equivalence point corresponded to a ratio 2.25. Similar studies were performed with the B form of the enzyme, which we have shown to contain one luciferase and one inactive luciferin (Henry et al., 1975), instead of the free luciferase (A form) (Figure 6). This complex, which is isolated during purification of the enzyme, is quite stable. Preincubation of the complex with luciferin for 5 min at 0 °C (7.3 pmol of complex) gave a stoichiometry of 0.92 molecules of luciferin in the complex luciferase-luciferin-inactive luciferin. Thus, in the final complex the luciferase is bound to one molecule of active luciferin (as shown by light emission by a pH jump to pH 9.0) and one molecule of inactive luciferin. Preincubation for a longer period (30 min at 0 °C) gave a plateau at 11.8 pmol of luciferin for 7.3 pmol of luciferase; that is, the ratio of active luciferin per molecule of luciferase is 1.62. Thus, under these conditions

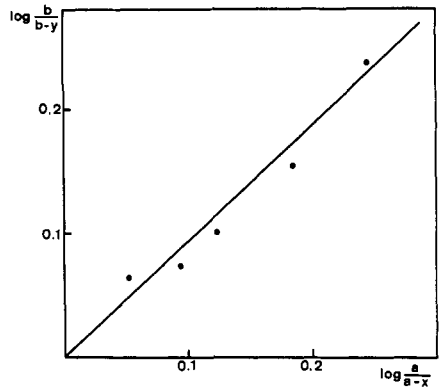

FIGURE 7: Inhibition by oxyluciferin of luciferin-luciferase association. To mixtures of luciferin (30.9 nM) and of oxyluciferin at various concentrations (7-112 nM) in 0.5 mL of 0.05 M acetate buffer (pH 4.8), luciferase (9.6 nM) was added and incubated at 0 °C for 60 s. The amounts of luciferin bound to the enzyme were then determined from measurements of the intensity of light emissions obtained after injection of the mixture into 2.5 mL of 0.1 M Tris-HCl buffer (pH 8.5) containing 0.5 M NaCl. It was assumed that after 60 s of incubation all the sites of luciferase were complexed and the displacement of oxyluciferin by luciferin was neglected. The ratio of the association rate constants was thus given by the equation: $k_a[I_{ox}]/k_a[I] = \log [b/(b-x)]/\log [a/(a-x)]$, where a and b were the initial concentrations of luciferin and oxyluciferin, respectively, and x was the concentration of bound luciferin at the end of the incubation period. The experimental slope was 0.94.

(preincubation at acidic pH), the inactive luciferin can be displaced from its site by active luciferin. The kinetics of displacement of inactive luciferin from the complex with luciferase could be readily followed. Preincubation of the complex luciferase-inactive luciferin (10 nM) with an excess of active luciferine (71 nM) at pH 4.8, followed by injection into buffer at pH 9.0, showed that, during an initial phase of 8 min at 0 °C, I_{max} gave a constant value but that further preincubation between 8 and 36 min resulted in an increase of I_{max}, this increase being first order. From the rate of increase with time (that is, displacement of inactive luciferin by active luciferin which is then oxidized with light emission), the half-time for this second displacement was determined to be 30 min (rate constant of 3.84×10^{-4} s^{-1}).

The above results indicate that two sites are available on the luciferase. These sites can be occupied by luciferin, inactive luciferin, or oxyluciferin. For luciferin, the determined values give an average figure of 2.44 ± 0.6 ($n = 7, p = 0.05$) for all the estimations. The two sites are equivalent and independent.

The kinetics of association of luciferin and luciferase at pH 4.8 were measured using the same approach. At 1 nM luciferase and 0.3 nM luciferin, association was studied between 10 and 30 s of preincubation and gave a value of $k_a = 9.6 \times 10^6$ M^{-1} s^{-1}. This figure is somewhat approximate owing to experimental difficulties; nevertheless, a second experiment with 0.1 nM luciferase and 0.3 nM luciferin using aliquots after 20 to 120 s of preincubation gave a value of $k_a = 3.6 \times 10^7$ M^{-1} s^{-1}. Thus, at least within an order of magnitude this constant can be accepted. These values are closely similar to that observed for the association of oxidized luciferin with luciferase at neutral pH. Indeed, when both luciferin and variable quantities of oxyluciferin were preincubated with luciferase to observe competition between oxyluciferin and luciferin (Vincent and Lazdunski, 1972), the ratio of the two association rate constants was found to be 0.94 (Figure 7).

Kinetics of dissociation of the luciferase-luciferin complex were studied by following displacement of luciferin from the complex luciferase-luciferin by oxyluciferin. A large excess of oxyluciferin was mixed with the preformed complex luciferase-luciferin to prevent reassociation of luciferase with dissociated luciferin. Inhibition of I_{max} (representative of the quantity of complex luciferase-luciferin remaining) was plotted as a semilog function against time of preincubation. However, even with a vry large excess of oxyluciferin (up to 500-fold) the results were not conclusive, since the half-time for dissociation of the complex luciferase-luciferin approaches that of the stability of the complex. It can nevertheless be considered that the half-lifetime is greater than 60 min.

The ratio of equilibrium dissociation constants for oxyluciferin and luciferin from the complex with luciferase was studied by displacement of oxyluciferin from a preformed complex with limiting quantities of luciferin. At zero time, both sites were occupied by oxyluciferin and after about 4 h the I_{max} increased from almost 0 to about 30% of the control incubation in absence of oxyluciferin. Total luciferin activity (as measured by treatment by $Fe^{2+}/O_2/PO_4^{3-}$) was maintained constant during the incubation by addition of fresh luciferin. Equilibrium was obtained after 8 h. The results may be interpreted by the series of equations shown below, where I is luciferin and I_{ox} is oxyluciferin.

$$AI_{ox_2} \rightleftharpoons AI_{ox} + I_{ox}$$

$$AI_{ox} + I \rightleftharpoons AII_{ox}$$

$$AII_{ox} \rightleftharpoons AI + I_{ox}$$

$$AI + I \rightleftharpoons AI_2$$

The results gave a very approximate value for $K_d[I_{ox}]/K_d[I]$ of about 1300.

Discussion

Recognition of luciferase as a copper protein and the efficient inhibition of the enzyme by diethyl dithiocarbamate have permitted elimination of a thermal denaturation treatment of the enzyme during purification of luciferin. Preparations of luciferin reported in this communication are about ten times more active than earlier described preparations (Henry et al., 1970). An absorption band at 307 nm is now easily visible (ϵ = 11 800) and during oxidation catalyzed by luciferase this band disappears. In the absence of any reducing agents, the enzyme oxidizes only two molecules of substrate and in this case no variation of the 307-nm band occurs, indicating that the spectral modification occurs after emission of light. This second step requires ascorbic acid or other reductants. For the moment, the mechanism is unknown but it may be supposed that the effects previously noted of dihydroxyfumaric acid, pyrogallol, catechol (Michelson and Isambert, 1973), and β-mercaptoethanol or reduced flavins (Henry et al., 1970) can be explained in a similar manner.

We have recently shown that luciferase contains about 10% sugars (Henry et al., 1975) and luciferin also contains an important sugar fraction, of approximately 20%. As shown in Table III, a certain similarity in composition of luciferin and luciferase is apparent. Owing to the peculiar characteristics of luciferase and luciferin, both of which are highly asymmetrical glycoproteins, the usual assumptions implied in determination of molecular weights cannot be applied. Thus, we now consider a more precise value for luciferin to be 34 000, rather than the earlier reported estimate of 45 000 (Henry et al., 1970, 1973).

Oxyluciferin can be purified by the same techniques as luciferin by the omission of diethyl dithiocarbamate. The two

TABLE IV: Proposed Kinetic and Thermodynamic Characteristics of the *Pholas* Protein-Protein Interactions.

	Luciferin-Luciferase Complex	Oxyluciferin-Luciferase Complex	B-Luciferase
Stoichiometry	2	2	1[a]
Dissociation equilibrium constant K_d (M)	1.2×10^{-11} [b]	1.7×10^{-8}	2.4×10^{-11} [b]
Association rate constant k_a (M^{-1} s^{-1})	1.6×10^{7} [c]	1.6×10^{7}	1.6×10^{7} [c]
Dissociation rate constant k_d (s^{-1})	1.9×10^{-4} [d]	0.27	3.8×10^{-4}
Half-lifetime $t_{1/2}$ (s^{-1})	3600	2.6	1800
$\Delta G_a°$ (kcal mol^{-1})		−10.6	
$\Delta H_a°$ (kcal mol^{-1})		+3.5	
$\Delta S_a°$ (cal mol^{-1} deg^{-1})		+47.3	

[a] Henry et al. (1975). [b] Obtained as k_d/k_a. [c] Assumed to be equal to k_a (I_{ox}). [d] Minimal value.

molecules have similar molecular weights and compositions in amino acids and sugars. Only the spectral and electrophoresis properties are different. Resolution of luciferin and oxyluciferin by electrophoresis in sodium dodecyl sulfate gels can be explained by a difference in charge of the two molecules.

Despite the very minor differences between luciferin and oxyluciferin, the two molecules form complexes with luciferase which are quite distinct. The stoichiometry of the complexes is two molecules of luciferin or oxyluciferin per molecule of luciferase. The low values obtained experimentally in the approach using iodinated oxyluciferin can be explained by the presence of small amounts of luciferase-inactive luciferin complex (B form) in the preparation of free luciferase (A form), whereas the high values obtained by titration can be explained by contamination of luciferin with small amounts of oxyluciferin. The enzyme thus contains two binding sites which are independent, as shown by the Scatchard plot of the association luciferase-oxyluciferin, by the presence of a single discontinuity in titration curves of luciferase with luciferin, and by the fact that when one site is blocked, as in form B of the enzyme, the activity is reduced by a factor of two. The presence of two sites is probably related to the dimeric nature of the enzyme, each subunit possessing an active site containing one atom of copper and capable of binding one molecule of substrate or of product. The complex oxyluciferin-luciferase has an equilibrium dissociation constant K_d of 17.2 nM and rate constants of association $k_a = 1.6 \times 10^7$ M^{-1} s^{-1} and of dissociation $k_d = 0.27$ s^{-1} at pH 7.5 and at 25 °C. Since under these conditions luciferin is rapidly oxidized by luciferase, the parameters of the luciferin-luciferase complex were determined at 0 °C and at pH 4.8. For technical reasons, the values thus obtained are less precise. The association rate constant is comparable to that of the oxyluciferin-luciferase complex, whereas dissociation is at least three orders of magnitude slower. The results are summarized in Table IV. The large differences in the equilibrium dissociation and dissociation rate constants are not explained by the different experimental conditions, as shown by the competition experiments which were performed to measure $k_a(I_{ox})/k_a(I)$ and $K_D(I_{ox})/K_d(I)$. In the same table, we present estimated values for the B form of luciferase (luciferase-inactive luciferin complex). Displacement of the inactive luciferin from this complex by luciferin gives an estimate of the rate of dissociation. The rate of association has been assumed to be essentially the same as that of the association of luciferase and luciferin. The values obtained show an interaction similar to that of luciferin, rather than oxyluciferin, and it is likely that this inactive form of the substrate present in small concentrations occupies statistically only one site on the enzyme.

The interaction of luciferase and oxyluciferin to form a complex can be compared with other known protein-protein interactions. Thermodynamically, the reaction has positive enthalpy and entropy of association and formation of the complex is an entropy-driven process. The high stability is presumably due to expulsion of water molecules (Lazdunski et al., 1974; Fulpius et al., 1972). In contrast, the association rate is high compared with those generally described. Thus, for the association of pancreatic secretory inhibitor with trypsin $k_a = 6.8 \times 10^6$ M^{-1} s^{-1} (Schweitz et al., 1973), while for the association of insulin with its receptor $k_a = 3.5 \times 10^6$ M^{-1} s^{-1} (Cuatrecasas et al., 1971). Similarly, association of haptoglobin with hemoglobin has a $k_a = 0.7 \times 10^6$ M^{-1} s^{-1} (Nagel and Gibson, 1971), while association of cobra toxin with the acetylcholine receptor has $k_a = 0.17 \times 10^6$ M^{-1} s^{-1} (Fulpius et al., 1972). Nevertheless, the value found for the association luciferase-oxyluciferin $k_a = 16 \times 10^6$ M^{-1} s^{-1} is not unreasonable: a value of 7000×10^6 M^{-1} s^{-1} has been determined for the association of the lactose repressor with the operator (Riggs et al., 1970). The value for oxyluciferin-luciferase approaches diffusion rates and it is thus possible that the reaction is controlled by diffusion. The role of the sugar residues in this rapid reaction will be explored in subsequent work.

The complex oxyluciferin-luciferase is also characterized by a high value for the dissociation rate. The half-lifetime is 2.6 s comparable with the value obtained for the association of *lac* repressor-operator ($t_{1/2}$ = 100 s) (Riggs et al., 1970) or for the association of tetrodoxin-garfish olfactory nerve membrane ($t_{1/2}$ = 44 s) (Henderson and Wang, 1972), but very different from those obtained for the association of pancreatic secretory inhibitor with trypsin ($t_{1/2}$ = 50 min) (Schweitz et al., 1973) or pancreatic trypsin inhibitor with trypsin ($t_{1/2}$ = 17 weeks) (Vincent and Lazdunski, 1972). In contrast to the complex oxyluciferin-luciferase, the half-lifetime for the complex with luciferin is about 60 min at pH 4.8 and 0 °C. This value approaches those obtained with protease inhibitors. It has been suggested that these high values are a result of the perfect fit of tertiary structures which occurs via numerous weak interactions (Vincent et al., 1974). It is evident that this kind of association prevents the enzyme from playing a truly catalytic role. Dissociation of the complex is facilitated by the existence of a secondary reaction which modifies the product. It may be noted that in the case of the association of the pancreatic trypsin inhibitor with trypsin modification of a single bond in the inhibitor (reduction of the disulfide bridge Cys-14-Cys-38) reduces the half-life of the complex by a factor of 10 000 (Vincent and Lazdunski, 1972). The small differences between luciferin and oxyluciferin might thus be responsible for the very large difference in half-lifetime for the

corresponding complexes (approximately 1000). The mechanism of the second modification occurring after emission of light would be of interest as a model system of the control of protein–receptor site interactions or other systems involving extremely high affinities coupled, nevertheless, with a reversibility.

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