Some Properties of Luciferase from the Bioluminescent Crustacean, *Cypridina hilgendorfii*†

Frederick I. Tsuji,* Richard V. Lynch, III,‡ and Charles L. Stevens

ABSTRACT: Luciferase from the bioluminescent ostracod crustacean, *Cypridina hilgendorfii*, was purified by fractional precipitation with acetone and ammonium sulfate, followed by DEAE-cellulose chromatography and preparative acrylamide gel electrophoresis. The enzyme was then studied by disc gel electrophoresis, gel elution chromatography, velocity and equilibrium sedimentation, and amino acid analysis. The molecular weight of luciferase was found to be approximately 68,000 by gel elution chromatography and equilibrium sedimentation. Solutions of luciferase examined were usually polydisperse. The subunit molecular weight was estimated to be about 13,700 by amino acid analysis, 11,500 by sodium dodecyl sulfate acrylamide gel electrophoresis, and 10,000 by equilibrium sedimentation. Luciferase lacked free sulfhydryl groups and a variety of potential inhibitors had little or no effect on the activity of the enzyme.

Cypridina hilgendorfii is a small (about 3 mm long) marine ostracod crustacean, found along the south coast of Japan. When disturbed, it produces a brilliant luminous cloud by ejecting luciferin (substrate) and luciferase (enzyme) from separate glands into sea water (Harvey, 1952). The luciferin is oxidized by molecular oxygen, catalyzed by luciferase, *via* the formation and decomposition of a four-membered peroxide (dioxetane) ring, yielding as products equimolar amounts of oxyluciferin and CO_2, plus light (λ_{max} 462 nm) (Stone, 1968; Shimomura *et al.*, 1969; Shimomura and Johnson, 1970, 1971). The oxygen of CO_2 is derived largely from molecular oxygen (Shimomura and Johnson, 1971) and the emitter is the oxyluciferin–luciferase complex (Shimomura *et al.*, 1969). The structure of luciferin has been determined (Kishi *et al.*, 1966a,b) and the compound has been synthesized (Kishi *et al.*, 1966c, 1969). A biosynthetic mechanism has been recently proposed for *Cypridina* luciferin involving the cyclization of arginylisoleucyltryptophan(McCapra and Roth, 1972).

Although *Cypridina* luciferase has been purified and studied in some detail previously (Shimomura *et al.*, 1961; Tsuji and Sowinski, 1961), no substantial work has been done recently and considerable uncertainties still remain regarding the properties of the enzyme, *viz.*, molecular weight and subunit structure. The present study represents an attempt to resolve some of these questions and to inquire further into the nature of the enzyme.

Experimental Section

Materials

PCMB,[1] *N*-ethylmaleimide, *N*-methylmaleimide, ME, α,α'-dipyridyl, and Tris were from Sigma Chemical; acrylamide and *N,N'*-methylenebisacrylamide, Eastman Organic Chemicals; SDS, Fisher Scientific; Me_2SO, Crown Zellerbach; urea, Schwarz/Mann; SKF 525-A, Smith Kline and French Labs; DPEA and DPDA, Lilly Labs; ovalbumin, Pentex; chymotrypsinogen A, α-chymotrypsin, lysozyme, ribonuclease (beef pancreas), and trypsin, Worthington Biochemical; cytochrome *c* (horse heart), alcohol dehydrogenase, γ-globulin (human), myoglobin (horse heart), pepsin (hog stomach), and Coomassie Brilliant Blue R-250, Mann; Pronase (B grade), Calbiochem; DEAE-cellulose, Schleicher and Schuell; Bio-Gel P-150, Bio-Rad; Sephadex G-150, Pharmacia Fine Chemicals. Others not listed were of the best commercial grade available.

Cypridina organisms were collected near Tateyama, Japan, and were rapidly air-dried and stored in desiccated jars. Luciferin was purified by the method of Tsuji (1955), using absolute methanol in place of 1-butanol for the initial extraction (Tsuji *et al.*, 1970). Luciferase was purified by fractional precipitation with acetone and ammonium sulfate, followed by DEAE-cellulose chromatography (Tsuji and Sowinski, 1961). Final purification was carried out by preparative acrylamide gel electrophoresis, as described below. The active fractions were combined, dialyzed against water, and freeze-dried. Some preparative acrylamide gel electrophoresis fractions were concentrated using a pressure ultrafilter (Amicon Diaflo) and others were examined directly. Deionized, glass distilled water was used throughout.

Methods

Preparative Acrylamide Gel Electrophoresis. The procedure of Jovin *et al.* (1964) was used; 100 mg of luciferase was dissolved in 5 ml of the prescribed upper buffer (Tris-glycine) containing 10% sucrose and layered over the upper gel. The electrophoresis was run approximately 18 hr at a constant current of 27 mA and 4°. The flow rate of the elution buffer was regulated with a finger pump at 0.3 ml/min and 7-ml fractions were collected. Each fraction was read at

† From the Department of Biological Sciences, University of Southern California, Los Angeles, California 90007, the Brentwood V. A. Hospital, Los Angeles, California 90073, and the Department of Biophysics and Microbiology, University of Pittsburgh, Pittsburgh, Pennsylvania 15260. *Received January 24, 1974.* This work was supported by research Grant GB-36017X from the National Science Foundation.

‡ Present address: Naval Research Laboratory, Washington, D. C. 20375.

[1] Abbreviations used are: PCMB, *p*-chloromercuribenzoic acid; SDS, sodium dodecyl sulfate; SKF 525-A, 2-diethylaminoethyl-2,2-diphenylvalerate hydrochloride; DPEA, 2,4-dichloro(6-phenylphenoxy)-ethylamine; DPDA, 2,4-dichloro-6-phenylphenoxyethyldiethylamine; Tris, tris(hydroxymethyl)aminomethane; ME, 2-mercaptoethanol.

280 nm and assayed for luciferase activity.

Disc gel electrophoresis was performed in a commercial apparatus (Hoefer Scientific Inst.) according to Weber and Osborn (1969), using 10% gel. Luciferase concentration ranged between 1 and 3 mg/ml and the concentrations of the other proteins were between 0.2 and 1.2 mg/ml. The gels were stained with Coomassie Brilliant Blue. One set of luciferase gels was left unstained, sliced into 2-mm thick discs, homogenized in 0.1 M Tris (pH 7.4), and assayed for luciferase activity. Molecular weight determinations were carried out in 1% SDS and ME, using as standards bovine serum albumin, ovalbumin, alcohol dehydrogenase, pepsin, chymotrypsinogen A, trypsin, α-chymotrypsin, lysozyme, ribonuclease, and cytochrome *c* (Weber and Osborn, 1969).

Gel Filtration. Molecular weight determinations were performed using calibrated columns (1.5 × 90 cm) of Bio-Gel P-150 and Sephadex G-150 (Andrews, 1964); 2–6 mg of luciferase or protein was eluted with 0.07 M sodium phosphate buffer (pH 6.8), and 2-ml fractions were collected. Void volume was determined with blue dextran. Elution curves were plotted from absorbance readings at 280 nm and also from activity measurements in the case of luciferase. Calibration curves were obtained by plotting peak elution volumes.

Protein Determination. Protein concentration was determined by the method of Lowry *et al.* (1951), using bovine serum albumin as a standard.

Absorbance Measurement. A Gilford spectrophotometer was used for routine absorbance measurements and a Zeiss PMQ II spectrophotometer for titrating sulfhydryl groups.

Sulfhydryl Group Determination. Titration of free sulfhydryl groups was carried out using standardized solutions of PCMB (Benesch and Benesch, 1962). Luciferase concentration was 0.50 mg/ml in 5×10^{-2} M Tris (pH 7.20). The luciferase was dialyzed against Tris buffer or denatured with 8 M urea overnight before being titrated. Samples were allowed to mix for 15 min following each addition of PCMB before being read at 250 nm.

Test of Potential Inhibitors. The monooxygenase inhibitors SKF 525-A, DPEA, and DPDA were dissolved in about 1 ml of Me_2SO and diluted with 0.1 M phosphate (pH 7.0) to a final concentration of 6.25×10^{-4} M, 1.25×10^{-3} M, and 6.25×10^{-4} M, respectively; 2 ml of each inhibitor solution was diluted with 1 ml of 0.1 M phosphate (pH 7.0) and mixed with 1 ml of luciferase solution (2×10^{-3} mg/ml). The control consisted of the same mixture without the inhibitor, but with the Me_2SO added. *N*-Ethylmaleimide, *N*-methylmaleimide, and iodoacetamide were incubated with luciferase (4×10^{-3} mg/ml) at a concentration of 0.1, 0.1, and 2.5×10^{-2} M, respectively, in 0.1 M Tris (pH 7.2). Sodium azide and α,α-dipyridyl were incubated with luciferase (4×10^{-3} mg/ml) at a concentration of 0.1 and 1.25×10^{-2} M, respectively, in 0.2 M Tris (pH 7.2). Pronase was incubated with luciferase (0.125 mg/ml) at a concentration ranging from 2.5×10^{-3} to 1.25×10^{-2} mg/ml in 0.2 M Tris (pH 8.0). After 1 hr all of the above mixtures were assayed for luciferase activity. SDS was incubated with luciferase (4×10^{-3} mg/ml) at a concentration ranging from 1×10^{-6} to 1×10^{-2} M in 0.1 M Tris (pH 7.2). After 30 min, the mixtures were assayed for luciferase activity.

Membrane Filtration. The peak luciferase fractions from preparative acrylamide gel electrophoresis were each concentrated with a pressure ultrafilter (Amicon Diaflo cell, UM 10 membrane with cut-off of mol wt 10,000) immediately prior to analysis. For the sedimentation experiments, the luciferase was transferred from its original solvent into 0.1 M Tris (pH 7.2) containing 0.1 M NaCl, by successively filtering and diluting with the solvent.

Sedimentation Velocity and Sedimentation Equilibrium. The experiments were carried out in a Spinco Model E analytical ultracentrifuge. The solvent used was 0.1 M Tris (pH 7.2) containing 0.1 M NaCl, and the concentration of luciferase varied from 1 to 10 mg/ml. Velocity sedimentation was performed at 60,000 or 56,000 rpm at 6° in a single-sector cell. The sedimentation coefficients were corrected to water at 20°. Equilibrium experiments were performed at 44,000 or 26,000 rpm at 6° and analyzed according to Yphantis (1964). Equilibrium was assumed to have been reached when the fringes of two successive photographs taken at least 12 hr apart showed no change. Concentration of solute at each radial position in the cell was calculated from measurements of fringe displacement in millimeters. The conversion factor was calculated from the wavelength of light used, the optical path length in the double-sector cell, the interfringe spacing, and the specific refractive increment of solute, dn/dc. The value of dn/dc at 546 nm was assumed to be 1.84×10^{-4} ml/mg, a value typical of proteins.

Luciferase Assay. To 1.0 ml of luciferase, 2.2 ml of luciferin was injected (Tsuji *et al.*, 1970). The luciferin solution was prepared by mixing 0.2 ml of an acidified (0.3 N HCl) stock solution of luciferin and 2.0 ml of 0.1 M Tris (pH 7.2). Fractions possessing high luciferase activity were diluted with 0.1 M Tris (pH 7.2) before being assayed. The initial light intensity (expressed in arbitrary light units) was determined with a MacNichol photomultiplier photometer (Chase, 1960) and served as a measure of activity.

Amino Acid Analysis. A Spinco Model 120C automatic amino acid analyzer was used for the analyses (Spackman *et al.*, 1958). Luciferase (1.0 mg) was hydrolyzed in 6 N HCl for 24, 48, and 72 hr (Moore and Stein, 1963). Tryptophan was determined by heating luciferase for 24 hr in 6 N HCl containing 4% thioglycolic acid (Matsubara and Sasaki, 1969). Performic acid oxidation was carried out by mixing 9.50 ml of 100% formic acid and 0.50 ml of 30% H_2O_2 at room temperature for 2 hr (solution A). The sample of luciferase (1.0 mg) was then dissolved in 300 μl of 100% formic acid (solution B). Both A and B were cooled in an ice bath and then 600 μl of A was added to 300 μl of B. The mixture was allowed to cool in an ice bath for 2.5 hr, and then diluted 1:10 with water and lyophilized. All analyses were carried out in duplicate.

Results

Preparative Acrylamide Gel Electrophoresis. Figure 1 shows the elution diagram of luciferase (second peak) following preparative acrylamide gel electrophoresis. The first peak, which preceded luciferase, was colored brown, whereas the third peak, which followed luciferase, was colorless. Both peaks were inactive with luciferin. Luciferase yielded a white powder on freeze-drying. However, in some runs, luciferase fractions were tinged brown, in which case the freeze-dried powder was also colored slightly brown. The specific activity of the luciferase was 500–800 times that of the starting *Cypridina* powder. No apparent association was found between color and activity. When the preparative acrylamide gel electrophoresis purified luciferase (100 mg) was subjected to a second preparative acrylamide gel electrophoresis, a similar elution pattern was observed. If the

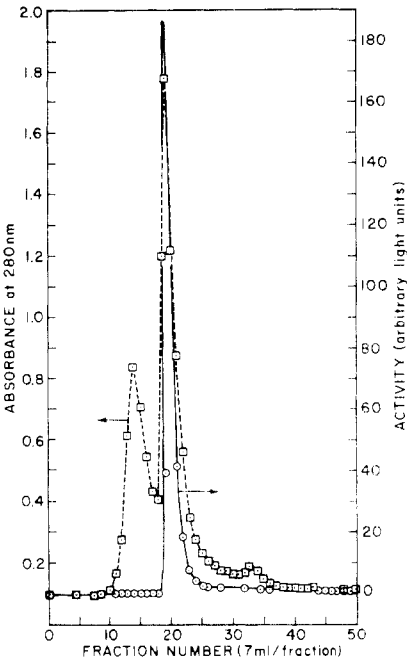

FIGURE 1: Elution pattern of *Cypridina* luciferase (middle peak) from preparative acrylamide gel electrophoresis. Luciferase was first purified by fractional precipitation with acetone and ammonium sulfate, followed by DEAE-cellulose chromatography. Flow rate of the elution buffer, 0.3 ml/min.

luciferase peak was colored slightly brown during the first run, the luciferase peak now became colorless. However, no significant increase in specific activity occurred. Luciferase gave a protein absorption spectrum with a maximum at 275 nm and $E_{1cm}(1\%)$ 7.628. In 5×10^{-2} M phosphate buffer, the pH–activity curve of luciferase was bell-shaped between pH 5.6 and 8.2, with a maximum at pH 7.1.

Disc Gel Electrophoresis. Luciferase gave two close bands on disc gel electrophoresis. The major band was luciferase, whereas the minor, faster moving, band was inactive with luciferin. Luciferase moved at almost the same rate as bovine serum albumin. Interestingly, luciferase stained with great difficulty. If luciferase was heated in 0.1% SDS and run in gels containing 0.1% SDS (Weber and Osborn, 1969), two bands were observed. The molecular weight of the major and minor bands were approximately 23,000 and 45,000, respectively. When luciferase was heated with 1% SDS and run in gels containing 1% SDS, only a single band was observed. The molecular weight was estimated to be about 11,500.

Gel Filtration. Columns of Bio-Gel P-150 and Sephadex G-150 were calibrated with myoglobin, trypsin, chymotrypsinogen A, ovalbumin, bovine serum albumin, and γ-globulin, using as the eluent 0.07 M phosphate buffer (pH 7.2). Both columns gave a value of approximately 68,000 for the molecular weight of luciferase. The position of luciferase in the elution diagram was very close to that of bovine serum albumin.

If luciferase was passed through a Bio-Gel P-150 column, three absorbance peaks were obtained: a main luciferase peak (second), a small peak which eluted ahead of luciferase (immediately following the void volume), and a third peak which eluted after luciferase. Only the second peak was active. If luciferase was again passed through the Bio-Gel column, the same pattern of three peaks was obtained. The absorbance readings for each peak, however, were lower and luciferase activity was significantly less. If the same procedure was repeated a third time, a similar result was obtained. Successive losses in activity occurred with each run. If the preparative acrylamide gel electrophoresis fractions containing luciferase were concentrated with a pressure ultrafilter (Amicon Diaflo, UM 10 membrane with cut-off of mol wt 10,000) and eluted through a Bio-Gel P-150 column, only the first and second (luciferase) peaks were found in the elution diagram.

Sulfhydryl Group Titration. Titration of luciferase and luciferase denatured with urea was performed using 1.22×10^{-3} and 1.22×10^{-4} M PCMB. No free sulfhydryl groups were detected.

Test of Potential Inhibitors. SKF 525-A, DPEA, and DPDA did not inhibit luciferase activity. These compounds have been previously shown to be inhibitors of monooxygenases (McMahon *et al.*, 1969; Hammond and White, 1970; Nealson and Hastings, 1972). *N*-Ethylmaleimide, *N*-methylmaleimide, iodoacetamide, and PCMB had little or no effect on luciferase activity, indicating that free sulfhydryl groups are not involved with activity. Sodium azide and α,α′-dipyridyl showed only little inhibitory activity, suggesting that the transition metals of group IB and IIB, such as iron, copper, and zinc, are probably not involved (Vallee and Wacker, 1970). Incubation with Pronase caused only a slight drop in luciferase activity. Luciferase, however, was strongly inhibited by SDS. The first drop in activity occurred at around 10^{-5} M SDS and the second drop at slightly above 10^{-4} M SDS. At concentrations above 10^{-3} M SDS, luciferase was completely inactivated.

Velocity and Equilibrium Sedimentation. For the velocity sedimentation experiments, two samples of luciferase were used. The first was purified as described in Methods, with the active fractions being combined, dialyzed, and freeze-dried. The second sample was purified similarly, except that the sample was subjected to preparative acrylamide gel electrophoresis twice and the active fractions were concentrated by membrane filtration without dialyzing. Both preparations had small amounts of turbid materials present. On one velocity run, the centrifuge was operated at 24,000 rpm. A minor, fast moving peak of about 18 S or 19 S was seen in some of the dilutions.

In the velocity runs, the samples were routinely diluted with the solvent to 2/3 and 1/3 of the original concentration (approximately 6 mg/ml). The first sample showed two sedimenting boundaries with $s_{w,20}^0$ values of 4.08 S and 1.36 S. The slower moving peak, however, contained only a small fraction of the total amount of material. The sedimentation rate of the faster peak showed very little or perhaps a small positive dependence on concentration. It is expected that proteins at this concentration and pH would show a marked negative dependence of s on concentration. The minor (second) peak showed a more normal dependence.

The second sample was run twice. The first time it was run at the initial concentration (approximately 8 mg/ml) and 2/3 and 1/3 dilution. Subsequently, the solutions were pooled and concentrated with a membrane ultrafilter to approximately the same volume as the initial sample, aliquots diluted by factors of 2/3 and 1/3 and run again. Both samples yielded a single sedimentation boundary and virtually the same sedimentation coefficients at the respective dilutions. The $s_{w,20}^0$ was 2.85 S and the plotted curve showed a strong positive dependence of s on concentration. This is characteristic behavior of particles made up of subunits

which then fragment or dissociate upon dilution. One cannot tell from these data whether these fragments are in a reversible chemical equilibrium or not.

The results of the sedimentation equilibrium experiments are given in Figure 2. Both number and weight average molecular weight of the preparations are plotted as a function of concentration at a specific radial position in the cell. The ratio of the two averages is a measure of the dispersity of the distribution. The error bars represent the variation in molecular weight appropriate to the root-mean-square deviation of the data points about the least-squares fit. Where error bars do not appear, the variation is within the limits of the symbol.

The data of Figure 2 were collected from two samples of luciferase. The first sample was prepared in the same way as the first sample in the sedimentation velocity experiment. The open triangles represent weight average and the filled ones represent number average molecular weight of the most dilute sample. Both averages extrapolate to about 10,000 molecular weight with an error of about 2000. At higher concentrations, the weight average increases and the increase is somewhat more than that of the number average. This indicates that the molecular weight of the smallest species is 10,000 ± 2,000, but the material is not homogeneous. That is, it contains some species of molecular weight greater than 10,000. It is possible that this component is the same as the slower moving component in the first sample of the velocity sedimentation experiment. The data which appear here were from the highest dilution (about 0.33 mg/ml); two experiments for which the loading concentration was about 0.67 and 1 mg/ml could not be analyzed because the concentration of solute at the meniscus was not zero. This means that there is a larger molecular weight species present which is not observed upon dilution, which agrees with the sedimentation velocity results.

The second luciferase sample was an aliquot of the second sample used for the velocity sedimentation experiment. The results are given in the upper part of Figure 2. Again, the sample was loaded at the initial concentration (approximately 1 mg/ml) and also diluted by a factor of 2/3 and 1/3. The upper set of points represent the highest concentration. The molecular weights at low point values of concentration (near the meniscus) are not reliable in this particular run since apparent molecular weight increases near the meniscus. Such aberrations are sometimes observed in experiments of this type (Yphantis, 1964). They usually arise from the difficulty in establishing the very low concentration values, but become successively less important as concentration increases toward the bottom of the cell; it appears that this material is rather homogeneous with a molecular weight of 68,000 ± 3000.

The sample loaded at intermediate concentration is somewhat less homogeneous. The molecular weight of the smallest species is about 40,000 or less with a rather wide limit on the low side. Molecular weight increases toward the bottom of the cell, rather sharply at first, and still increases beyond 65,000. This is not inconsistent with the simultaneous presence of species with a molecular weight 70,000 and 35,000.

Data for the aliquot loaded at 1/3 concentration were virtually the same as the 2/3 data plotted.

Finally, the sedimentation equilibrium experiments were repeated using preparative acrylamide gel electrophoresis fractions taken from the elution peak and stored frozen until needed. The fractions were not concentrated by membrane filtration or by freeze-drying, but used directly after thawing. The loading concentration on one run was 0.14 mg/ml and 0.20 mg/ml on another. The results were virtually the same as indicated by the squares of Figure 2.

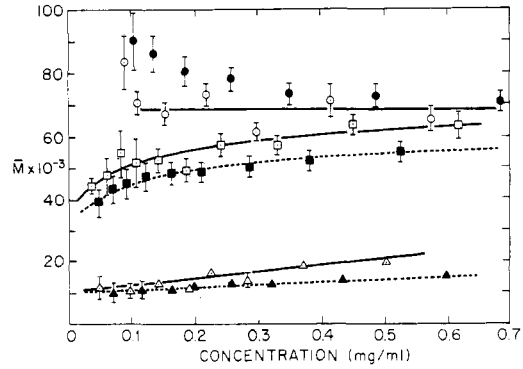

FIGURE 2: Sedimentation equilibrium of luciferase. The centrifuge speed was 44,000 (△) or 26,000 rpm (○ and □) at 6°. The initial concentration of luciferase was about 1 mg/ml in 0.1 M Tris (pH 7.2) containing 0.1 M NaCl. Solutions were loaded in the centrifuge cell at this concentration (circles) or diluted in buffer by a factor of ⅔ (squares) or by ⅓ (triangles). The open and closed symbols represent weight and number average molecular weights, respectively. Concentration on the abscissa represents point values of concentration in the cell at equilibrium. The triangles represent data from the first sample and the remaining symbols, data from the second. See Methods for other details.

TABLE I: Amino Acid Composition of *Cypridina* Luciferase as Compared to the Known Composition of *Renilla* Luciferase.

Amino Acid	Residues/Luciferase Molecule: Nearest Integer[a]	Residues/Luciferase Molecule: *Renilla* Luciferase[b]
Aspartic acid	16	14
Threonine	9	6
Serine	6	6
Glutamic acid	19	14
Glycine	9	8
Alanine	7	7
Valine	8	6
Methionine	2	2
Isoleucine	6	5
Leucine	7	8
Tyrosine	4	3
Phenylalanine	5	4
Half-cystine	6	3
Lysine	8	8
Histidine	1	2
Arginine	4	3
Tryptophan	0	2
Proline	6	5

[a] Based on molecular weight of 11,500 as determined by SDS acrylamide gel electrophoresis. [b] Based on molecular weight of 12,000 as determined by SDS acrylamide gel electrophoresis and gel elution chromatography in 8 M urea (Karkhanis and Cormier, 1971).

Amino Acid Composition. Table I shows the amino acid composition of luciferase. The number of amino acid residues is based on assuming a molecular weight of approxi-

mately 11,500, as determined by acrylamide gel electrophoresis. The molecular weight, calculated from the amino acid composition, was 13,692. The number of residues was calculated by taking an average of the 24-, 48-, and 72-hr values and rounding off to the nearest integer. Except for threonine and serine, no significant destruction of amino acids occurred and recovery of added norleucine was satisfactory. In the case of threonine and serine, the 24-hr values were used. Basic hydrolysis did not indicate the presence of any tryptophan. Performic acid oxidation and subsequent analyses revealed the presence of two methionine and six cysteic acid residues, indicating the presence of three disulfide bonds from three cystines in the enzyme. The analyses also revealed the presence of one residue each of glucosamine and galactosamine. The apparent partial specific volume, calculated from the amino acid composition (Cohn and Edsall, 1943), was 0.719 cm^3/g.

Discussion

The present results, based on calibrated gel columns and sedimentation equilibrium, indicate that the molecular weight of luciferase is 68,000 ± 3000. In contrast, values previously reported, based on sedimentation-viscosity, have been 35,300, 35,700 and 39,000 (Chase, 1955), and 79,650 (Tsuji and Sowinski, 1961), and those based on sedimentation-diffusion have been 70,000 (Chase and Langridge, 1960), 48,500 and 53,000 (Shimomura *et al.*, 1961), and between 52,000 and 57,000 (Shimomura *et al.*, 1969). An upper limit of 80,000, based on the diffusion coefficient, has also been reported (Fedden and Chase, 1959). The molecular weight obtained from osmotic pressure has been approximately 80,000, whereas that obtained from light scattering has been well above 100,000 (F.I. Tsuji, unpublished results).

The sedimentation coefficient, extrapolated to zero concentration, appears to be around 4.08 S. Other published values (measured in different solvents) are: 2.89 S, 3.44 S, 3.56 S (Chase, 1955), 5.3 S (Chase and Langridge, 1960), 4.58 S (extrapolated to zero concentration) (Tsuji and Sowinski, 1961), 4.3 S (Shimomura *et al.*, 1961), 4.66 S (extrapolated to zero concentration) (Shimomura *et al.*, 1969), and 3.72 S (Stone, 1968). Velocity sedimentation also showed a small peak with a sedimentation coefficient of 1.36 S.

The sedimentation data indicate that luciferase fragments or dissociates upon dilution. This is shown both by the dependence of $s_{w,20}$ on concentration, the existence of a lowered average molecular weight, and a more disperse system upon dilution (Figure 2). Further, it is likely that this dissociation is not reversible at least under the conditions of our equilibrium experiments. If it were reversible, we would obtain identical dependence of the molecular weight averages on point values of concentration regardless of the loading concentration (Yphantis, 1964). Although the 2/3 and 1/3 concentrations gave the same result, these appear to differ with the result for the sample run at full concentration. It is obvious that the results of the first equilibrium run are quite different (the triangles of Figure 2). If this material were known to be a subunit of luciferase, it would show unequivocally that the fragments or subunits were not in association–disassociation equilibrium.

The results also suggest that luciferase has a tendency to breakdown during gel elution chromatography. This is indicated by the formation of a new peak with a molecular weight of around 10,000 or less with each successive chromatographic runs. However, attempts to restore activity have been unsuccessful, including the addition of calcium ions which have been found to be essential for activity (Lynch *et al.*, 1972). Luciferase has also been reported to be rapidly and irreversibly inactivated by high concentrations of urea (Osborne and Chase, 1954; Strasburger *et al.*, 1964; Chase, 1966).

Luciferase appears to be an acidic protein. The content of aspartic and glutamic acids (Table I) is high in relation to the content of arginine and lysine. These results are consistent with the low values of 3.28 (Weir *et al.*, 1955) and 4.35 (Shimomura *et al.*, 1961) previously reported for the isoelectric point. The apparent partial specific volume of 0.719 cm^3/g, calculated from the amino acid composition (Table I), is also in reasonable agreement with the value of 0.707 cm^3/g determined experimentally (Tsuji and Sowinski, 1961).

Since luciferase gave only a single band on acrylamide gel electrophoresis in 1% SDS, it would seem that the enzyme possesses only one subunit species. The amino acid analysis gave a value of approximately 13,700 for the molecular weight of the smallest luciferase unit. This value is somewhat higher than the value of 11,500 and 10,000, obtained from SDS acrylamide gel electrophoresis and sedimentation equilibrium, respectively. It seems therefore that the true value is probably somewhere between these high and low values, possibly in the neighborhood of 12,000. The observed value of 68,000 for the molecular weight of native luciferase suggests that the enzyme exists as some multiple of this unit, possibly a hexamer.

There are apparent similarities between *Cypridina* luciferase and *Renilla* (coelenterate) luciferase (Karkhanis and Cormier, 1971), especially with respect to amino acid composition (Table I). The content of cystine is twice that of *Renilla* luciferase and tryptophan is absent. The content of threonine, valine, aspartic acid, and glutamic acid is also much higher than in *Renilla* luciferase. Aside from these differences, the amino acid composition of *Cypridina* luciferase does not vary by more than one residue from that of *Renilla* luciferase. This is interesting because the two animals are so widely separated in the evolutionary tree. Further, it is also interesting to note that the luciferins (substrates) of *Cypridina* and *Renilla* are rather similar, both possess a pyrazine ring in their basic structures (Hori and Cormier, 1973). The bioluminescence reactions of both animals liberate CO_2 as a product and the source of carbon of the CO_2 is the same carbonyl group (Shimomura and Johnson, 1971; DeLuca *et al.*, 1971). The luciferins differ in their side groups and apparently in the mechanism by which oxygen is incorporated into CO_2. The source of oxygen in the case of *Cypridina* is largely molecular oxygen (Shimomura and Johnson, 1971), whereas in the case of *Renilla*, it is water (DeLuca *et al.*, 1971).

Acknowledgments

We thank Dr. Anthony T. Barnes and Mrs. Nora Barnes of the University of California, Santa Barbara, and Dr. Michael A. Raftery and Mr. John Racs of the California Institute of Technology, for the amino acid analyses. The SKF 525-A was kindly supplied by Dr. Glenn E. Ullyot of Smith Kline and French Laboratories; DPEA and DPDA were the gift of Dr. Robert E. McMahon of Lilly Research Laboratories. We also thank Mr. David H. Donald and Miss Sanda Werther for technical assistance.

References

Andrews, P. (1964), *Biochem. J. 91,* 222.

Benesch, R., and Benesch, R. E. (1962), *Methods Biochem. Anal. 10,* 43.

Chase, A. M. (1955), *J. Cell. Comp. Physiol. 45,* 13.

Chase, A. M. (1960), *Methods Biochem. Anal. 8,* 61.

Chase, A. M. (1966), *in* Bioluminescence in Progress, Johnson, F. H., and Haneda, Y., Ed., Princeton, N. J., Princeton University Press, p 115.

Chase, A. M., and Langridge, R. (1960), *Arch. Biochem. Biophys. 88,* 294.

Cohn, E. J., and Edsall, J. T. (1943), Proteins, Amino Acids and Peptides, New York, N. Y., Reinhold, p 375.

DeLuca, M., Dempsey, M. E., Hori, K., Wampler, J. E., and Cormier, M. J. (1971), *Proc. Nat. Acad. Sci. U. S. 68,* 1658.

Fedden, G. A., and Chase, A. M. (1959), *Biochim. Biophys. Acta 32,* 176.

Hammond, R. K., and White, D. C. (1970), *J. Bacteriol. 103,* 607.

Harvey, E. N. (1952), Bioluminescence, New York, N. Y., Academic Press, p 297.

Hori, K., and Cormier, M. J. (1973), *Proc. Nat. Acad. Sci. U. S. 70,* 120.

Jovin, T., Chrambach, A., and Naughton, M. A. (1964), *Anal. Biochem. 9,* 351.

Karkhanis, Y. D., and Cormier, M. J. (1971), *Biochemistry 10,* 317.

Kishi, Y., Goto, T., Hirata, Y., Shimomura, O., and Johnson, F. H. (1966a), *Tetrahedron Lett.* 3427.

Kishi, Y., Goto, T., Hirata, Y., Shimomura, O., and Johnson, F. H. (1966b), *in* Bioluminescence in Progress, Johnson, F. H., and Haneda, Y., Ed., Princeton, N. J., Princeton University Press, p 89.

Kishi, Y., Goto, T., Inoue, S., Sugiura, S., and Kishimoto, H. (1966c), *Tetrahedron Lett.,* 3445.

Kishi, Y., Sugiura, S., Inoue, S., and Goto, T. (1969), *Yakugaku Zasshi 89,* 1657.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193,* 265.

Lynch, R. V., III, Tsuji, F. I., and Donald, D. H. (1972), *Biochem. Biophys. Res. Commun. 46,* 1544.

Matsubara, H., and Sasaki, R. M. (1969), *Biochem. Biophys. Res. Commun. 35,* 175.

McCapra, F., and Roth, M. (1972), *J. Chem. Soc., Chem. Commun.,* 894.

McMahon, R. E., Mills, J., Culp, H. W., Gibson, W. R., Miller, W. R., and Marshall, F. J. (1969), *J. Med. Chem. 12,* 207.

Moore, S., and Stein, W. H. (1963), *Methods Enzymol. 6,* 819.

Nealson, K. H., and Hastings, J. W. (1972), *J. Biol. Chem. 247,* 888.

Osborne, A. W., and Chase, A. M. (1954), *J. Cell. Comp. Physiol. 44,* 49.

Shimomura, O., and Johnson, F. H. (1970), *Photochem. Photobiol. 12,* 291.

Shimomura, O., and Johnson, F. H. (1971), *Biochem. Biophys. Res. Commun. 44,* 340.

Shimomura, O., Johnson, F. H., and Masugi, T. (1969), *Science 164,* 1299.

Shimomura, O., Johnson, F. H., and Saiga, Y. (1961), *J. Cell. Comp. Physiol. 58,* 113.

Spackman, D. H., Stein, W. H., and Moore, S. (1958), *Anal. Chem. 30,* 1190.

Stone, H. (1968), *Biochem. Biophys. Res. Commun. 31,* 386.

Strasburger, A. K., Schweizer, R. D., and Chase, A. M. (1964), *Amer. Zool. 4,* 424.

Tsuji, F. I. (1955), *Arch. Biochem. Biophys. 59,* 452.

Tsuji, F. I., Lynch, R. V., III, and Haneda, Y. (1970), *Biol. Bull. 139,* 386.

Tsuji, F. I., and Sowinski, R. (1961), *J. Cell. Comp. Physiol. 58,* 125.

Vallee, B. L., and Wacker, W. E. C. (1970), *Proteins 5,* 129.

Weber, K., and Osborn, M. (1969), *J. Biol. Chem. 244,* 4406.

Weir, J. H., Tsuji, F. I., and Chase, A. M. (1955), *Arch. Biochem. Biophys. 56,* 235.

Yphantis, D. A. (1964), *Biochemistry 3,* 297.