Kim, S., and Paik, W. K. (1970), *J. Biol. Chem. 245,* 1806–1813.
Lee, H. W., and Paik, W. K. (1972), *Biochim. Biophys. Acta 277,* 107–116.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193,* 265–275.
McIlwain, H. (1966), in Biochemistry and the Central Nervous System, Boston, Mass., Little Brown and Co., pp 270–299.
Miyake, M. (1975), *J. Neurochem. 24,* 909–915.
Ogawa, Y., Quagliarotti, G., Jordan, J., Taylor, C. W., Starbuck, W. C., and Busch, H. (1969), *J. Biol. Chem. 244,* 4387–4392.
Paik, W. K. and Kim, S. (1968), *J. Biol. Chem. 243,* 2108–2114.
Paik, W. K., and Kim, S. (1971), *Science 174,* 114–119.
Paik, W. K., and Kim, S. (1973), *Biochim. Biophys. Acta 313,* 181–189.
Paik, W. K., and Kim, S. (1975a), *Adv. Enzymol. 42,* 227–286.
Paik, W. K., and Kim, S. (1975b), *FEBSBY 34,* 127–135.
Paik, W. K., Kim, S., Ezirike, J., and Morris, H. P. (1975), *Cancer Res. 35,* 1159–1163.
Paik, W. K., Kim, S., and Lee, H. W. (1972b), *Biochem. Biophys. Res. Commun. 46,* 933–941.
Paik, W. K., Lee, H. W., and Morris, H. P. (1972a), *Cancer Res. 32,* 37–40.
Reporter, M., and Corbin, J. L. (1971), *Biochem. Biophys. Res. Commun. 43,* 644–650.
Salvatore, F., Zappia, V., and Shapiro, S. K. (1968), *Biochim. Biophys. Acta 158,* 461–464.
Sundarraj, N., and Pfeiffer, S. E. (1973), *Biochem. Biophys. Res. Commun. 52,* 1039–1045.
Tanford, C. (1962), *J. Am. Chem. Soc. 84,* 4240–4247.
Vesterberg, O. (1971), *Methods Enzymol. 22,* 389–412.
Wilkinson, G. N. (1961), *Biochem. J. 80,* 324–332.

Purification and Properties of *Renilla reniformis* Luciferase†

John C. Matthews,‡ Kazuo Hori, and Milton J. Cormier*

ABSTRACT: Luciferase from the anthozoan coelenterate *Renilla reniformis* (*Renilla* luciferin:oxygen 2-oxidoreductase (decarboxylating), EC 1.13.12.5.) catalyzes the bioluminescent oxidation of *Renilla* luciferin producing light (λ_B 480 nm, Q_B 5.5%), oxyluciferin, and CO_2 (Hori, K., Wampler, J. E., Matthews, J. C., and Cormier, M. J. (1973), *Biochemistry 12,* 4463). Using a combination of ion-exchange, molecular-sieve, sulfhydryl-exchange, and affinity chromatography, luciferase has been purified, approximately 12 000-fold with 24% recovery, to homogeneity as judged by analysis with disc and sodium dodecyl sulfate-polyacrylamide gel electrophoresis, gel filtration, and ultracentrifugation. *Renilla* luciferase is active as a nearly spherical single polypeptide chain monomer of 3.5×10^4 daltons having a specific activity of 1.8×10^{15} $h\nu$ s^{-1} mg^{-1} and a turnover number of 111 μmol min^{-1} μmol^{-1} of enzyme. This enzyme has a high content of aromatic and hydrophobic amino acids such that it has an $\epsilon_{280nm}^{0.1\%}$ of 2.1 and an average hydrophobicity of 1200 cal $residue^{-1}$. The high average hydrophobicity of luciferase, which places it among the more hydrophobic proteins reported, is believed to account, at least in part, for its tendency to self-associate forming inactive dimers and higher molecular weight species.

The chemistry of the bioluminescent oxidation of a fully active synthetic analogue of *Renilla* luciferin by *Renilla* luciferase (*Renilla* luciferin:oxygen 2-oxidoreductase(decarboxylating), EC 1.13.12.5.) and the identity of the product excited state of this reaction are now known (Hori et al., 1973). The reaction path is illustrated in Figure 1.

Enzymological and structural studies on *Renilla* luciferase were not possible in the past due to isolation procedures which led to mixtures of active and inactive luciferase. Earlier preparations also apparently contained high levels of a single contaminating protein which was incorrectly characterized as luciferase (Karkhanis and Cormier, 1971). We have now overcome these difficulties partly by necessary changes in the initial handling and processing of the animals and partly by improved isolation procedures which include an affinity chromatography step. We report the isolation of *Renilla* luciferase, purified 12 000-fold to homogeneity, and the reexamination of the properties of the enzyme. In addition, we provide evidence that *Renilla* luciferase exists in its active form as a single polypeptide chain of 35 000 daltons and that this enzyme can self-associate to inactive higher molecular weight species.

Materials and Methods

Materials. Methanol and dimethylformamide were spectrophotometric or equivalent grade and, unless otherwise specified, all other chemicals used were reagent grade or the best quality available. All buffers and solutions were made up using deionized water or water twice distilled from glass having a maximum conductivity of 1.2 $\mu\Omega^{-1}$. DEAE[1]-cellulose was

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[1] Abbreviations used are: 2-ME, 2-mercaptoethanol; BSA, bovine serum albumin; Nbs_2, 5,5′-dithiobis(2-nitrobenzoate); DEAE, diethylaminoethyl; BSA, bovine serum albumin; DMF, dimethylformamide; EDTA, (ethylenedinitrilo)tetraacetic acid; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol; TEMED, *N,N,N′,N′*,-tetramethylethylenediamine.

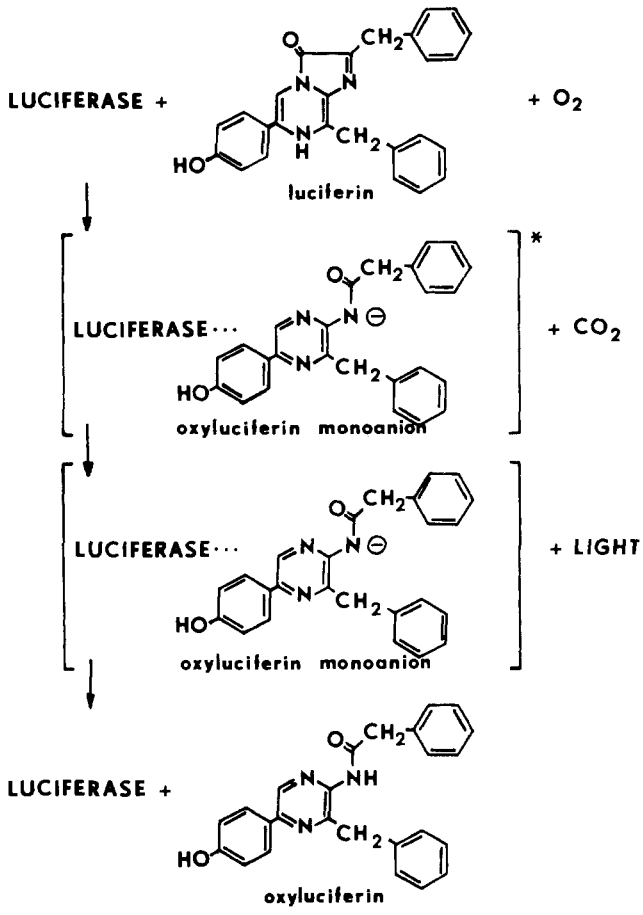

FIGURE 1: Chemical path to light emission during *Renilla* in vitro bioluminescence.

Whatman DE-22, *p*-benzyloxyaniline was from ICN Pharmaceuticals, and acrylamide, methylenebisacrylamide, and Temed were electrophoresis grade from Bio-Rad. Proteins used as chromatography and electrophoresis standards were the best grades available from Sigma. Synthetic luciferin and oxyluciferin (Figure 1) were prepared as previously described (Hori et al., 1973).

Luminescence Assays. A portable photometer, designed and constructed by the University of Georgia Bioluminescence Laboratory, was used for routine assays. Its capabilities include the simultaneous digital readout measure of peak intensity (via a peak hold circuit), total photons over a predetermined time period, and light intensity as a function of time by use of an external recorder. The instrument was calibrated in absolute units by use of the luminol light standard (Lee et al., 1966). Bioluminescence emission spectra were obtained and corrected using an on-line spectrophotofluorimeter system previously described (Wampler et al., 1971; Wampler and DeSa, 1971).

Luciferase assays were performed by mixing 25 pmol of luciferin, dissolved in 10 μl of methanol made 1 M in HCl, with 1 ml of luciferase in assay buffer (0.5 M NaCl, 0.1 M potassium phosphate, 1.0 mM Na_2EDTA, 0.02%, w/v, BSA, 0.6 mM NaN_3, pH 7.6) at 25 °C. Peak intensity was taken as a measure of initial rate. Total light assays were determined by injecting 1.0 ml of luciferase, dissolved in assay buffer, into 10 μl of a methanolic-HCl solution of luciferin.

Preparation of Column Resins. Nbs_2-Sepharose was prepared by coupling Nbs_2 to diaminohexane-Sepharose according to the method of Lin and Foster (1975). Diaminohexane-Sepharose was prepared from Sepharose 4B-200 and 1,6-diaminohexane by the method of March et al. (1974). *p*-Benzyloxyaniline-Sepharose was prepared by coupling 10 g of *p*-benzyloxyaniline to 500 ml of Sepharose 4B-200 in 50% DMF using the procedure described by Cuatrecasas (1970).

Purification of Luciferase. All steps were carried out at 4 °C and all buffers contained 0.6 mM NaN_3 to reduce the incidence of bacterial proteases, which rapidly inactivate luciferase. *Renilla* were processed as described by Anderson et al. (1974), quick frozen in liquid N_2, and stored at −85 °C. Storage at −85 °C was necessary, since storage of the animals at −20 °C resulted in loss of luciferase activity. The average processed animal weighs 1.2 g, contains 0.1–0.2 nmol of luciferase, and requires 9.6 ml of extraction buffer for optimal extraction of the enzyme. For these reasons, the luciferase concentration in crude extracts is low. In addition luciferase in crude preparations loses 10–20% of its activity per day prior to chromatography on Sephadex G-100, a treatment which stabilizes the enzyme. In order to overcome these problems of large volume and instability of the enzyme, it has been necessary to devise a purification procedure which, in its early stages, allows rapid and effective concentration and purification of large volumes of crude extract.

Renilla Extraction (Step 1). A total of 6 kg of frozen animals was extracted in 300-g batches in 2.4 l. of 1.5 mM Tris base–1.0 mM Na_2EDTA an pH 7.8 (extraction buffer) by homogenizing for 1 min at maximum speed with a Tekmar, Model SD-45, homogenizer. The homogenates were centrifuged at 8000*g* for 5 min, resulting in a total supernatant volume of 48 l.

First DEAE (Step 2). Luciferase from 12 l. of the supernatant from step 1 was adsorbed onto a DEAE-cellulose column (14.6 × 6 cm) preequilibrated with extraction buffer. The column was washed with 10 l. of extraction buffer containing 35 mM NaCl prior to elution of luciferase by 15 mM Tris base–10 mM Na_2EDTA at pH 7.5. Four such DEAE columns were utilized simultaneously in order to rapidly handle the initial 48 l. of supernatant.

Second DEAE (Step 3). The combined luciferase fractions from step 2 were adjusted to pH 9.2 with 10 N NaOH and then adsorbed to a 14.6 × 18 cm DEAE column preequilibrated with extraction buffer. Luciferase was then eluted with 7.5 mM Tris base–0.75 M NaCl–5 mM Na_2EDTA at pH 7.0.

Sephadex Chromatography (Step 4). Combined luciferase fractions from step 3 were passed through a 14.6 × 90 cm Sephadex G-100 (40–120 micromesh) column which was preequilibrated with azide-free extraction buffer. Azide was omitted, since luciferase elutes prematurely on Nbs_2-Sepharose columns in the presence of this compound.

Nbs_2-Sepharose Chromatography (Step 5). The combined luciferase fractions from step 4 were applied to Nbs_2-Sepharose (2.4 × 22 cm) which had been preequilibrated with azide-free extraction buffer. The column was washed with 2 l. of 10 mM 2-ME in azide-free extraction buffer. Although luciferase contains free SH groups, it does not elute from the column at this stage. However, many other SH-containing proteins are eluted under these conditions. Luciferase was subsequently eluted from the column with a 5-l. linear gradient from 0 to 0.5 M NaCl in azide-free extraction buffer.

p-Benzyloxyaniline-Sepharose Chromatography (Step 6). Combined luciferase fractions from step 5 were applied to a *p*-benzyloxyaniline-Sepharose column (3.4 × 17 cm) which had been preequilibrated with extraction buffer. The column was washed with 2 l. of 7.5 mM Tris base–1 M NaCl–5 mM Na_2EDTA at pH 7.8 and luciferase was then eluted by using the same buffer made 35% in ethylene glycol.

Desalting and Reconcentration (Step 7). Combined luciferase fractions from step 6 were chromatographed on Sephadex G-100 (40–120 micromesh), as described for step 4, to remove the ethylene glycol. The active luciferase fractions were then concentrated by adsorbtion to a DEAE column (1 × 15 cm) preequilibrated with extraction buffer and the luciferase was eluted with 7.5 mM Tris base–0.75 M NaCl–5 mM Na_2EDTA at pH 7.0. DEAE was used routinely during the purification as a concentration step, since other methods, such as precipitation by salt, gave poor recoveries.

Sephadex Chromatography (Step 8). The combined luciferase fractions from step 7 were passed through a 2.9 × 145 cm column of Sephadex G-75 (10–40 micromesh) which had been preequilibrated with extraction buffer. The constant specific activity fractions across the major portion of the peak were judged to be homogeneous by several criteria (see Results). Subsequent rechromatography of the constant specific activity fractions on Sephadex G-75 (10–40 micromesh) or on ion-exchange columns did not result in increased specific activity.

Electrophoretic Analyses. Disc polyacrylamide gel electrophoresis was carried out at 4 °C at pH 8.0 and 9.5 according to the methods of Gabriel (1971). Polyacrylamide gels (10%) with 1% cross-linkage were used and up to 100 μg of protein was applied to each gel (5 ×100 mm).

The method of Neville (1971) was used for sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Polyacrylamide gels (15%) with 1% cross-linkage were used. BSA, catalase, yeast enolase, ovalbumin, carbonic anhydrase, trypsin inhibitor, lysozyme, cytochrome *c*, ribonuclease A, and luciferase were preincubated in a mixture of sodium doecyl sulfate (1%) and 2-ME (1%) for 1 h followed by heating at 80 °C for 10 min. This was followed by dialysis vs. a mixture of sodium dodecyl sulfate (0.1%) and 2-ME (1%) for 24 h prior to electrophoresis using 2 μg of each protein.

Gels were stained for protein localization with Coomasie brilliant blue G-250 in 10% perchloric acid according to the method of Reisner et al. (1975). Luciferase activity in pH 8.0 system disc gels was correlated with protein position by using one half of the gel for staining and the other half for assay purposes. One millimeter sections were assayed by crushing them in 1.0 ml of assay buffer and assaying for luciferase activity as described above.

Ultracentrifugation Studies. Sedimentation coefficients for luciferase were determined by the method of Schachman (1957) and high-speed sedimentation equilibrium experiments were done by the short-column technique of Yphantis (1964) using a Spinco, Model E, analytical ultracentrifuge.

Gel-Filtration Analyses. A 1.7 × 145 cm Sephadex G-75 (10–40 micromesh) column, equilibrated at 4 °C, with 0.1 M KCl–10 mM Tris base–1 mM Na_2EDTa at pH 7.5, was used to determine the Stokes' radius and the gel-filtration molecular weight of luciferase. Catalase and $NaNO_2$ were used to determine the void and salt volumes of the column, respectively, and the column was calibrated with BSA, ovalbumin, carbonic anhydrase, trypsin inhibitor, cytochrome *c*, and ribonuclease A. Elution positions for each protein were determined by measuring the absorbance at 280 nm for each 1.4-ml fraction. The Stokes' radius of luciferase was determined from its elution position by the method of Akers (1967). The molecular weight of luciferase, based on gel filtration, was determined by comparing its elution volume to a plot of the log molecular weight of the standard proteins vs. their elution volumes. The frictional ratio of luciferase was calculated from values obtained for its Stokes' radius, partial specific volume, and an average molecular weight determined from sedimentation equilibrium and velocity data.

Amino Acid and Carbohydrate Analyses. These were carried out on a Spinco, Model 120-C, automatic amino acid analyzer according to the method of Spackman et al. (1958). Hydrolysates were prepared by heating luciferase under vacuum in 6 *N* HCl at 110 °C. Most amino acid determinations were from 22-, 48-, and 72-h hydrolysates of 1.0 mg of luciferase by the method of Moore and Stein (1963). Tryptophan was determined by hydrolyzing 0.5 mg of luciferase, for 22 h, in the presence of 4% thioglycolate according to the method of Matsubara and Sasaki (1969). Cysteine was determined as cysteic acid from 22-h hydrolysates of 0.5 mg of performic acid oxidized luciferase prepared according to the method of Moore (1963). Cysteine was also determined as free sulfhydryls by titrating the 6 N guanidine-HCl denatured enzyme with Nbs_2 according to the method of Ellman (1959). The $\epsilon_{280nm}^{0.1\%}$ for luciferase was determined using crystalline BSA as standard with the biuret reaction according to Gornall et al. (1949).

Amino sugars were determined on the amino acid analyzer from 0.75 mg of luciferase hydrolyzed at 100 °C for 22 h in 3 M *p*-toluenesulfonic acid (Allen and Neuberger, 1975). Total carbohydrate content of luciferase was determined, using glucose as a standard, with the phenol–sulfuric acid reaction (Dubois et al., 1956).

Results

Luciferase Purification. The results of luciferase purification are summarized in Table I. This enzyme has been purified 12 000-fold, to a specific activity of approximately 1.8×10^{15} $h\nu\ s^{-1}\ mg^{-1}$, with 24% overall recovery. The enzyme turns over in vitro under optimum assay conditions with a turnover number of 111 $\mu mol\ min^{-1}\ \mu mol^{-1}$ luciferase exhibiting an apparent Michaelis constant for synthetic luciferin of 0.21 μM in air equilibrated buffer. We believe that luciferase is homogeneous, since it migrates as a single staining component during both disc and sodium dodecyl sulfate–polyacrylamide gel electrophoresis. In disc gels, the single stained band corresponds with the location of the activity. Further, luciferase sediments with a single symmetrical boundary during sedimentation velocity experiments and the relationship of the natural log of protein concentration vs. the square of the radius of rotation at sedimentation equilibrium is linear.

Amino Acid and Carbohydrate Composition. The amino acid composition of luciferase is presented in Table II. Note the abundance of hydrophobic and aromatic amino acid residues. Performic acid oxidation of luciferase gave a half-cystine content of 3.3 residues/35 000 daltons and titration of guanidine hydrochloride denatured luciferase with NbS_2 (see Methods) yielded 2.9 free sulfhydryls/35 000 daltons. Thus, we conclude that native luciferase contains 3 free SH groups but no disulfide linkages. Luciferase contains approximately 3% carbohydrate by weight which consists, in part, of glucosamine and galactosamine.

Physicochemical Data. Table III is a list of physicochemical constants determined for luciferase from various techniques. Based on these data, we have determined that native luciferase exists as a relatively symmetrical, single polypeptide chain having a molecular weight of 35 000 daltons. The enzyme is active as a monomeric species, since the subunit molecular weight based on sodium dodecyl sulfate–polyacrylamide gel electrophoresis is in good agreement with the molecular weight values determined for native luciferase.

Luciferase exhibits an absorbance maximum at 280 nm ($\epsilon_{280nm}^{0.1\%} = 2.1$) and a shoulder at 290 nm, as shown in Figure

TABLE I: Summary of Luciferase Purification.

Step[a]	Vol (ml)	Protein (mg)	Act. $h\nu\ S^{-1} \times 10^{14}$	Sp Act.	Purification	Yield (%)
Extraction (step 1)	48 000	170 000	251	0.00148		100
First DEAE (step 2)	32 000	6 270	234	0.0373	25.2	93
Second DEAE (step 3)	2 040	1 780	185	0.104	70.3	74
Sephadex (step 4)	4 250	1 340	151	0.113	76.4	60
Nbs_2-Sepharose (step 5)	1 400	287	130	0.453	306	52
PBA[b]-Sepharose (step 6)	1 690	55.4	121	2.18	1 470	48
Third DEAE (step 7)	6.50	20.3	97.3	4.97	3 360	39
Sephadex (step 8)	17.6	3.39	60.4	17.8	12 000	24

[a] See methods. [b] *p*-Benzyloxyaniline.

TABLE II: Amino Acid Composition of Luciferase.

Amino Acids	Residues/ 35 000 g[a]	Nearest Integer
Lysine	25.9	26
Histidine	9.5	10
Arginine	12.3	12
Aspartic acid	30.6	31
Threonine	8.5[b]	9
Serine	19.6[b]	20
Glutamic acid	36.0	36
Proline	17.3	17
Glycine	18.9	19
Alanine	19.2	19
Valine	22.9[c]	23
Methionine	6.9	7
Isoleucine	19.5[c]	20
Leucine	23.0	23
Tyrosine	11.5	12
Phenylalanine	15.1	15
Tryptophan	6.8[d]	7
Half-cystine	3.3[e]	3

[a] Unless otherwise indicated, these values represent an average of those obtained at 24, 48, and 72 h. Based on a molecular weight of 35 000 daltons as determined by sodium dodecyl sulfate gel electrophoresis and sedimentation equilibrium data. [b] Extrapolated to zero hydrolysis time. [c] Taken from the 72-h value. [d] Determined by hydrolyzing in the presence of thioglycolate (see Methods). [e] Determined as cysteic acid (see Methods).

TABLE III: Physicochemical Data on Luciferase.

Stokes' radius, a (Å)	25.5
Frictional ratio, f/f_0	1.2
Sedimentation coefficient $S^0_{20,w} \times 10^{13}$ (S)	3.1
Apparent partial specific volume $\bar{V}_{app}$ ($cm^3\ g^{-1}$)	0.74
Extinction coefficient, $\epsilon_{280nm}^{0.1\%}$	2.1
Average hydrophobicity (cal res^{-1})	1 200
Molecular weight	
from sedimentation equilibrium	33 000 ± 2000
from sedimentation velocity	38 000 ± 2000
from gel filtration	33 000 ± 2000
from sodium dodecyl sulfate electrophoresis molecular weight average	37 000 ± 4000 35 000

2. There is no detectable absorption or fluorescence in the visible region in purified preparations of luciferase. The corrected in vitro bioluminescence emission spectrum (Figure 3) is broad with an emission maximum near 480 nm and a low-intensity shoulder near 400 nm. The bioluminescence quantum yield for synthetic luciferin, based on the luminol standard (Lee et al., 1966), is approximately 5.5% and the luciferase-catalyzed oxidation of 1 mol of luciferin consumes approximately 1 mol of oxygen, as determined with a Rank O_2 electrode.

Effects of Environmental Factors and Inhibitors. The temperature optimum for luciferase is 32 °C and the pH optimum is 7.4. The enzyme will withstand incubation at temperatures up to 45 °C for 1 h without loss in activity and it is stable within a pH range from 6 to 10. Activity is stimulated by the presence of salts, such as NaCl or KCl, with optimum stimulation of 2.7-fold at 0.5 M. The addition of extraneous proteins also enhanced luciferase activity to varying degrees. BSA was the most effective of the proteins tested and for this reason it was included in the assay mixture. The optimum concentration of BSA was 0.2 mg/ml at which a 2.3-fold increase in luciferase activity was observed.

Luciferase is inhibited approximately 50% by the following divalent cations: Zn^{2+} and Cu^{2+}, 10^{-5} M; Fe^{2+}, 10^{-4} M; Mn^{2+}, 10^{-3} M; Ca^{2+}, 5×10^{-2} M; Sr^{2+}, 10^{-1} M. These inhibitions were completely reversible with Na_2EDTA. Mg^{2+} did not inhibit at a concentration of 0.3 M.

p-Benzyloxyaniline is a competitive inhibitor of luciferase having a K_i of 7.7 μM. We have taken advantage of this property to develop the *p*-benzyloxyaniline-Sepharose affinity column for use in luciferase purification. Oxyluciferin (Figure 1), a product of the bioluminescence reaction, is also a strong competitive inhibitor of luciferase having a K_i of 23 nM.

Evidence for Self-Association. Luciferase is quite stable when stored in dilute solution (<0.5 mg/ml) at 4 °C in extraction buffer. Under these conditions, the enzyme loses less than 5% of its activity per month. When stored at higher concentrations, the enzyme slowly self-associates at rates which increase with increasing concentration. For example, when stored at a concentration of 1 mg/ml, luciferase loses about 20% of its activity via self-association per day. This phenomenon does not appear to involve intermolecular disulfide bond

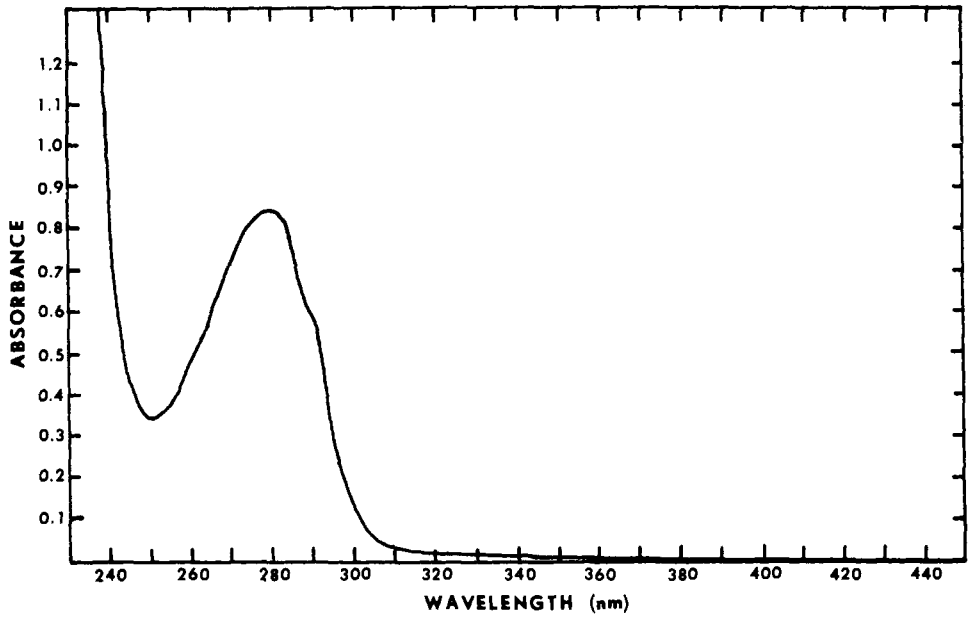

FIGURE 2: Absorbtion spectrum of luciferase.

formation, since incubation of the enzyme in 10 mM 2-ME has no effect on the rate of self-association. Luciferase preparations, which have become partially inactivated due to self-association, contain high-molecular-weight, inactive protein components. This can be demonstrated by rechromatography of such preparations on Sephadex (see Figure 4A). The percentage of higher molecular weight, inactive protein corresponds to the percentage of luciferase activity lost, while the specific activity of the recovered luciferase corresponds with that of freshly prepared enzyme. When self-associated luciferase fractions from several such preparations are pooled and rechromatographed on Sephadex (Figure 4B), dimers and higher multiple molecular weight forms of luciferase are apparent. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of self-associated luciferase produces a protein band identical to that obtained from native luciferase. However, attempts to regain active luciferase monomers from a mixture of higher molecular weight associated species have thus far failed.

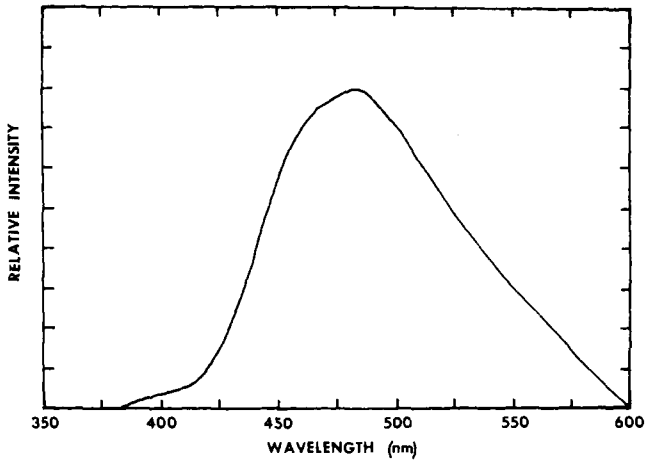

FIGURE 3: Bioluminescence emission of the luciferase-catalyzed oxidation of luciferin.

Discussion

Analysis of *Renilla* luciferase with disc and sodium dodecyl sulfate-polyacrylamide gel electrophoresis, gel filtration, and ultracentrifugation indicate that the enzyme is homogeneous and that it is active as a single polypeptide chain monomer of 35 000 daltons. In addition, it has not been possible to increase its specific activity of 1.8×10^{15} $h\nu$ s^{-1} mg^{-1} by any method tested. From amino acid composition and Nbs_2 titration data, we conclude that active luciferase contains 3 free sulfhydryl groups, but no disulfide linkages.

Luciferase also contains a relatively high proportion of hydrophobic amino acid residues, which give the enzyme an average hydrophobicity (Bigelow, 1967) of 1200 cal/residue. This places luciferase among the more hydrophobic proteins reported. Firefly luciferase is also known to be quite hydrophobic and its average hydrophobicity has been reported to be 1240 cal/residue (Denburg and McElroy, 1970). The high average hydrophobicity value for *Renilla* luciferase suggests an excess of hydrophobic residues such that some of these must reside on the surface of the protein (Fisher, 1964). Such an arrangement may lead to intermolecular hydrophobic bonding, which could, in part, account for the self-associating nature of this protein.

The shoulder near 400 nm observed in the bioluminescence emission spectrum (Figure 3) can be explained from our previous data on the nature of the product excited states during this reaction (Hori et al., 1973). The products of this reaction were shown to be oxyluciferin (Figure 1) and CO_2, while the product excited state, which accounts for the major band near 480 nm, is the monoanion of oxyluciferin (Figure 1). In aprotic solvents, the monoanion of oxyluciferin exhibits a fluorescence maximum near 470 nm, while the neutral species exhibits a fluorescence maximum near 400 nm (Hori et al., 1973). Thus, we suggest that the observed shoulder near 400 nm in the bioluminescence emission spectrum arises from the neutral species excited state. This contribution must be small, since the fluorescence quantum yield (ϕ_F) for the neutral species in those aprotic solvents which mimic the luciferase environment is 23% while that of the monoanion is about 6% (Hori et al., 1973; Ward and Cormier, 1976).

Our earlier estimate (Hori et al., 1973) that the excitation yield during *Renilla* bioluminescence approaches 100% is also

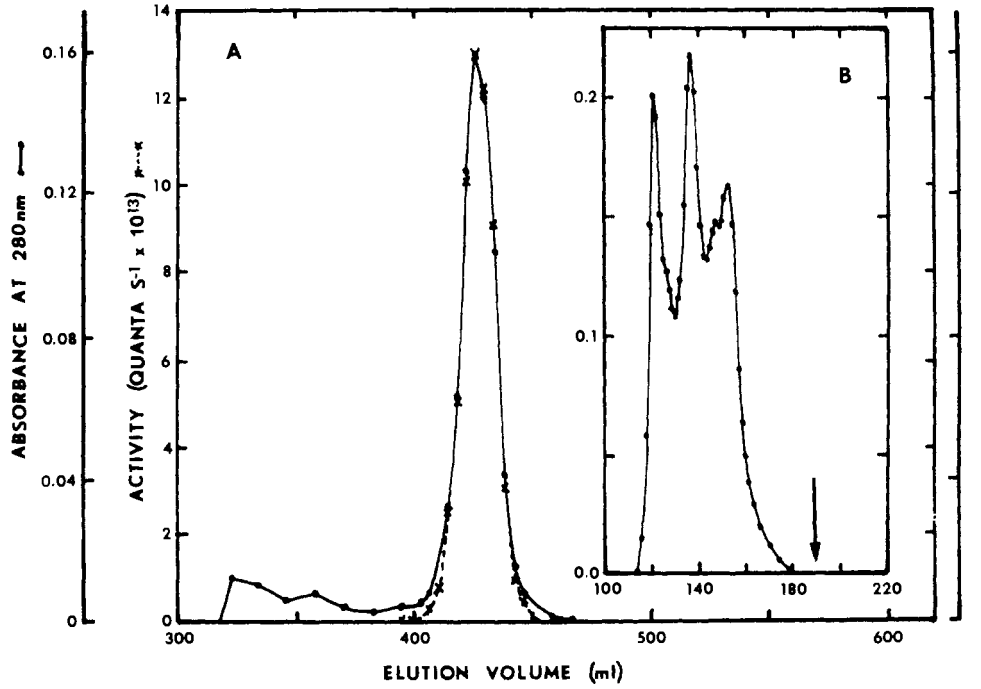

FIGURE 4: Sephadex chromatography of a mixture of native and self-associated luciferase (A). B represents rechromatography of the pooled, self-associated luciferase fractions. The arrow marks the elution position for native luciferase. For details of chromatography in A, see step 8 of "purification of luciferase" under Methods; for B see "gel filtration analyses" under Methods.

corroborated by recent experiments, which show that, although the bioluminescence quantum yield for luciferin in this reaction is only 5.5%, in vitro energy transfer to an acceptor (green fluorescent protein) approaches 100% (Ward and Cormier, 1976). Therefore, the excitation yield of the bioluminescence reaction must also approach 100%. From data of Hori et al. (1973), the excitation yield during chemiluminescence of luciferin in aprotic solvents has been calculated to be approximately 1.7%. Thus, *Renilla* luciferase increases the excitation yield approximately 60-fold during the luminescent oxidation of luciferin.

Although oxyluciferin, the product of the *Renilla* bioluminescence reaction, is a powerful competitive inhibitor of luciferase, we have been unable to observe a visible fluorescence from spent reaction mixtures or from mixtures of luciferase and oxyluciferin. Neither oxyluciferin nor its monoanion is fluorescent in water but they are highly fluorescent in aprotic solvents (Hori et al., 1973). From this and the above discussion, we suggest that the oxyluciferin monoanion product excited state resides in a hydrophobic enzyme environment which is different from that of the ground-state complex of luciferase and oxyluciferin.

It is of interest to compare *Cypridina* with *Renilla* bioluminescence, since the substrates, products, and in vitro reaction paths are analogous. The two luciferins differ only in the three substitutions on the fused imidazolone-pyrazine nucleus (Hori et al., 1975). During *Cypridina* in vitro bioluminescence, oxyluciferin readily forms a fluorescent complex with luciferase, which has the same spectral characteristics as the bioluminescence emission (Shimomura et al., 1969). This indicates that, unlike the *Renilla* system, the ground-state product in the *Cypridina* reaction resides in an environment on the enzyme essentially identical to its environment in the excited state. Furthermore, the dissociation constant for the oxyluciferin-luciferase complex in *Renilla* is lower than that in *Cypridina* by approximately an order of magnitude ($K_i = 2.3 \times 10^{-8}$ M for *Renilla* and $K_d = 3 \times 10^{-7}$ M for *Cypridina;* Shimomura et al., 1969). This difference in the dissociation constants for the reaction products from these two systems could, in part, explain the approximately tenfold difference in the catalytic rates of the two enzymes; 111 μmol min^{-1} μmol^{-1} of luciferase for *Renilla* and 1600 μmol min^{-1} μmol^{-1} of luciferase for *Cypridina* (Shimomura et al., 1969).

The production of light by calcium-triggered photoproteins, such as aequorin, is now known to involve a protein-bound chromophore that appears indistinguishable from *Renilla* luciferin (Hori et al., 1975; Ward and Cormier, 1975; Shimomura and Johnson, 1975). Further, it has been proposed that the mechanism of light production by both photoproteins and the *Renilla* luciferase-luciferin reaction is essentially identical (Hori et al., 1975; Ward and Cormier, 1975). *Renilla* luciferase and aequorin exhibit similar characteristics; i.e., they are both single polypeptide chain proteins and the reported molecular weight of 31 000 daltons for aequorin (Shimomura and Johnson, 1969) is near the molecular-weight value for *Renilla* luciferase reported here. It would be interesting to study possible structural homologies in these two proteins.

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References

Akers, G. K. (1967), *J. Biol. Chem. 242,* 3237.
Allen, K., and Neuberger, A. (1975), *FEBS Lett. 60,* 76.
Anderson, J. M., Charbonneau, H., and Cormier, M. J. (1974), *Biochemistry 13,* 1195.
Bigelow, C. C. (1967), *J. Theor. Biol. 16,* 187.
Cuatrecasas, P. (1970), *J. Biol. Chem. 245,* 3059.
Denburg, J. L., and McElroy, W. D. (1970), *Biochemistry 9,*

4619.
Dubois, M., Gilles, K. A., Hamilton, J. K., Rebens, P. A., and Smith, F. (1956), *Anal. Chem. 28,* 350.
Ellman, G. L. (1959), *Arch. Biochem. Biophys. 82,* 70.
Fisher, H. F. (1964), *Proc. Natl. Acad. Sci. U.S.A. 51,* 1285.
Gabriel, O. (1971), *Methods Enzymol. 22,* 565.
Gornall, A. G., Bardawill, C. J., and David, M. M. (1949), *J. Biol. Chem. 177,* 751.
Hori, K., Anderson, J. M., Ward, W. W., and Cormier, M. J. (1975), *Biochemistry 14,* 2371.
Hori, K., Wampler, J. E., Matthews, J. C., and Cormier, M. J. (1973), *Biochemistry 12,* 4463.
Karkhanis, Y. D., and Cormier, M. J. (1971), *Biochemistry 10,* 317.
Lee, J., Wesley, A. S., Fergurson, J. F., and Seliger, H. H. (1966), in Bioluminescence in Progress, Johnson, F. H., and Haneda, Y., Ed., Princeton, N.J., Princeton University Press, p 35.
Lin, L. J., and Foster, J. F. (1975), *Anal. Biochem, 63,* 485.
March, S. C., Parikh, I., and Cuatrecasas, P. (1974), *Anal. Biochem. 60,* 149.
Matsubara, H., and Sasaki, R. M. (1969), *Biochem. Biophys. Res. Commun. 35,* 175.
Moore, S. (1963), *J. Biol. Chem. 238,* 235.
Moore, S., and Stein, W. H. (1963), *Methods Enzymol. 6,* 819.
Neville, D. M. (1971), *J. Biol. Chem. 246,* 6328.
Reisner, A. H., Nemes, P., and Bucholtz, C. (1975), *Anal. Biochem. 64,* 509.
Schachman, H. K. (1957), *Methods Enzymol. 4,* 32.
Shimomura, O., and Johnson, F. H. (1969), *Biochemistry 8,* 3991.
Shimomura, O., and Johnson, F. H. (1975), *Nature (London) 256,* 236.
Shimomura, O., Johnson, F. H., and Masugi, T. (1969), *Science 162,* 1299.
Spackman, D. H., Stein, W. H., and Moore, S. (1958), *Anal. Chem. 30,* 1190.
Wampler, J. E., and DeSa, R. J. (1971), *Appl. Spectrosc. 25,* 623.
Wampler, J. E., Hori, K., Lee, J. W., and Cormier, M. J. (1971), *Biochemistry 10,* 2903.
Ward, W. W., and Cormier, M. J. (1975), *Proc. Natl. Acad. Sci. U.S.A. 72,* 2530.
Ward, W. W., and Cormier, M. J. (1976), *J. Phys. Chem. 80,* 2289.
Yphantis, D. A. (1964), *Biochemistry 3,* 297.

Oxidation of Spermidine and Spermine in Rat Liver: Purification and Properties of Polyamine Oxidase†

Erkki Hölttä

ABSTRACT: A novel enzyme responsible for the oxidation of spermidine and spermine has been found in rat liver. Spermidine is shown to be degraded to putrescine and 3-aminopropionaldehyde, and spermine to be cleaved to spermidine and 3-aminopropionaldehyde. A single enzyme catalyzing both reactions and designated as polyamine oxidase has been purified 4000-fold to electrophoretic homogeneity. Polyamine oxidase appears to be a flavoprotein, containing flavin adenine dinucleotide (FAD) as a prosthetic group. Hydrogen peroxide is evolved in the reaction and no other electron acceptors except molecular oxygen have been found. The molecular weight of the enzyme was approximately 60 000 and the sedimentation coefficient 4.5 S. The enzyme appears to be a single polypeptide chain since no evidence for structural subunits was obtained. Polyamine oxidase was sensitive to sulfhydryl and carbonyl group reagents. The optimum pH value for the oxidation of polyamines was close to 10. The reaction velocities were enhanced by various aldehydes, especially certain aromatic aldehydes. Polyamine oxidase appears to be localized in peroxisomes of liver cells, although the existence of an isoenzyme in the cytosolic fraction was not definitively ruled out. No marked changes were observed in the activity of polyamine oxidase in rat liver after partial hepatectomy, carbon tetrachloride poisoning, and after treatment with growth hormone or thioacetamide, conditions which are known to alter profoundly the metabolism and accumulation of polyamines.

The enzymic degradation of the polyamines spermidine and spermine in mammalian tissues is rather poorly understood. An exception is the amine oxidase in blood plasma of many ruminants, which acts on the primary amino groups of polyamines, and is fairly extensively studied and characterized. Brain of some mammalian species, connective tissue of bovine and chick aorta as well as bone are the other few tissues which reportedly contain polyamine oxidizing enzymes, probably similar to that found in ruminant plasma (Kapeller-Adler, 1970; Cohen, 1971; Tabor and Tabor, 1972; Bachrach, 1973).

In general, not much information is available of any other kind on the catabolism of spermidine and spermine in mammalian cells. Rosenthal and Tabor (1956) reported that 4–8% of parenterally administered spermine was excreted as spermidine into urine in the rat. Siimes (1967) observed some conversion of spermidine to putrescine, and spermine to spermidine in rat liver after injections of [^{14}C]polyamines to the animals. These findings in vivo were later confirmed and extended by Hölttä et al. (1973b). The hepatic conversion of [^{14}C]spermidine to labeled putrescine was markedly enhanced

† From the Department of Medical Chemistry, University of Helsinki, SF-00170 Helsinki 17, Finland. *Received June 24, 1976.*