Structurally Distinct Bacterial Luciferases*

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ABSTRACT: Bacterial luciferase catalyzes a bioluminescent oxidation of reduced flavin mononucleotide by molecular oxygen, the photon yield of the reaction being greatly stimulated by the presence of a long-chain aldehyde. Although luciferases from different bacterial strains have similar requirements, we have found a strain (designated as MAV) which, when compared with *Photobacterium fischeri*, has distinctive enzymatic differences in the quantitative responses to different reduced flavins and different aldehydes, in the pH–activity profile and in the kinetics of light emission. Structurally this MAV luciferase also has apparent similarities to the *Photobacterium fischeri* luciferase but even more distinctive differences. The molecular weights of both are about 80,000 with two different subunits of nearly but not exactly equal size.

The subunits are all distinctively different, judged both by their amino acid compositions and by the fact that no hybridization occurs between pairs from the different luciferases. It is assumed that the proteins are related and possess active-site structural similarities, studies of which should be of value in understanding the mechanism by which chemical energy is converted into light energy.

Bioluminescent bacteria emit a continuous and sometimes brilliant blue–green (490 nm) light. As deduced from the properties of purified bacterial luciferase, this emission occurs as a result of the bioluminescent oxidation of reduced flavin mononucleotide ($FMNH_2$) in the presence of a long-chain aldehyde (Hastings and Gibson, 1963). At the time luciferase was first isolated, Cormier and Strehler (1954) demonstrated that extracts of a variety of different luminous bacterial isolates had similar requirements for substrates. Although different bacterial strains are known to differ slightly in the color of the light emitted (Spruit and Van der Burg, 1950; McCloskey, 1960; Seliger and McElroy, 1965), no further analysis of differences between different species or strains has been reported.

The present paper reports on the isolation and characterization of a bacterial luciferase which differs markedly from the luciferase used in previous studies reported from this laboratory, which had been isolated from *Photobacterium fischeri*. The differences involve virtually all quantitative aspects of the luciferase: the aldehyde response and the kinetics of the light emission, the response of the luciferase to different reduced flavins, the pH–activity profile, and the structure of both subunits of the protein.

Although the ultrastructural features of the bacterium in which this variant enzyme occurs have not yet been compared with those of other strains, it seems desirable to report the properties of its luciferase. This enzyme is especially easy to purify, and it crystallizes readily. It is currently being used in a variety of studies, including structure analysis by X-ray diffraction (P. B. Sigler, personal communication) and amino acid sequence determination (R. L. Heinrickson, personal communication). Since it constitutes on the order of 4% of the soluble protein of the cell, it can readily be obtained in gram quantities.

These findings provide compelling documentation for adhering to the practice of using identifiable (and, when possible, readily available) strains. Luminous bacteria have too frequently been selected and/or described in the literature only by their light-emitting property. The fact that the bacterial luciferases can differ so extensively is of interest not only with regard to the origin, function, and evolution of the property of light emission (Seliger and McElroy, 1965); a comparison of their molecular structures by X-ray analysis should be especially relevant to our understanding of the biochemical mechanism whereby chemical energy is converted into light energy.

Materials and Methods

Photobacterium (*Achromobacter*) *fischeri* was obtained from the American Type Culture Collection (7744). Its luciferase (*P. fischeri*) was isolated and purified using procedures previously described (Hastings and Gibson, 1963; Friedland, 1968; Hastings *et al.*, 1965). The preparation of subunits was carried out as before by DEAE chromatography in 8 M urea (Friedland and Hastings, 1967a,b). Techniques for recombining the subunits to form the active renatured luciferase involved dilution into buffer, as in procedures previously used. Analytical disc gel electrophoresis was carried out in either dilute buffer or 8 M urea to study luciferase and its subunits, respectively (Friedland, 1968).

Pending its proper classification, the second strain of luminous bacteria has been given the laboratory designation "MAV." Although the origin of the strain is uncertain, the properties of the MAV luciferase are sufficiently different from those of the *P. fischeri* luciferase to assert that it did not arise by mutation(s) from *P. fischeri* in the laboratory. The strain was found in our culture collection, mislabeled *P. fischeri* 7744. A clue to its nature is that it will grow with nitrate as the sole source of nitrogen, similar to a strain studied by Coffey (1967). Unfortunately, the origin of his strain also appears uncertain; however, we did not possess a slant of Coffey's strain at the time the mislabeling occurred.

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TABLE I: Amino Acid Compositions of Luciferases *Photobacterium fischeri* and MAV and Their α- and β-Polypeptide Chains.[a]

	MAV α	MAV β	MAV	*P. fischeri*	*P. fischeri* α	*P. fischeri* β
Lysine	16.3	18.9	17.8	19.3	14.7	24.3
Histidine	13.1	10.4	11.6	9.6	8.8	10.6
Arginine	14.2	13.2	13.7	11.7	14.7	8.5
Aspartic acid	42.5	48.2	45.6	43.6	43.2	44.3
Threonine	17.4	21.8	19.8	25.4	28.4	22.2
Serine	22.9	17.0	19.8	19.3	17.6	21.1
Glutamic acid	46.9	39.7	43.1	45.6	44.1	47.5
Proline	9.8	12.3	11.2	10.6	10.8	10.6
Glycine	19.6	26.5	23.3	21.8	25.5	18.0
Alanine	36.0	26.5	30.9	20.8	20.6	21.1
Half-cystine	6.6	8.5	7.6	6.6	6.9	6.3
Valine	29.4	18.9	23.8	22.8	25.5	20.0
Methionine	9.8	8.5	9.1	9.6	10.8	8.5
Isoleucine	9.8	18.9	14.7	22.3	21.5	23.2
Leucine	25.1	27.4	26.4	23.9	20.6	27.5
Tyrosine	10.9	16.1	13.7	14.7	14.7	14.8
Phenylalanine	24.0	17.0	20.2	19.3	18.6	20.1
Tryptophan	2.2	4.7	3.6	3.6	4.9	2.1

[a] The results are expressed for all proteins as the number of moles of residues per 40,000 g.

The procedures for isolating and purifying the MAV luciferase, modified from those used for *P. fischeri*, are described below.

The molecular weights of the luciferase subunits (both those of *P. fischeri* and MAV) were determined by dodecyl sulfate electrophoresis on polyacrylamide gels following the procedure of Shapiro *et al.* (1967) as modified by Weber and Osborn (1969). The lyophilized proteins and their subunits were dissolved in 0.01 M sodium phosphate buffer (1% in sodium dodecyl sulfate and 1% in β-mercaptoethanol, pH 7.2) at a protein concentration of 0.5 mg ml^{-1}. After incubation for 1 hr at 40°, the solutions were dialyzed against 0.01 M sodium phosphate buffer (pH 7.2, 0.1% in dodecyl sulfate and 0.1% in β-mercaptoethanol) and then subjected to acrylamide gel electrophoresis as described by Weber and Osborn (1969). Molecular weight calibrations were carried out using protein standards.

The amino acid compositions were determined with luciferases of the highest purity available (see Figure 6). The two enzyme solutions were first passed through a Sephadex G-25 column, equilibrated with 0.05 M NH_4HCO_3, and then lyophilized. The separated α and β subunits were treated similarly. After exhaustive dialysis against 0.05 M NH_4HCO_3 to remove salts and urea, the solutions were dialyzed against distilled water and the subunits were recovered by lyophilization.

The dry proteins were hydrolyzed under a vacuum for 24, 48, and 72 hr in 6 M HCl at 108°. Amino acid analyses were performed by the method of Spackman *et al.* (1958) on a Spinco Model 116 amino acid analyzer. The values reported in Table I were obtained by averaging three duplicate sets of 22-hr hydrolysates of the different proteins. The number of micromoles was converted into residues per molecule assuming a molecular weight of 40,000 for all subunits and enzymes. The values for serine and threonine were obtained after linear extrapolation of the number of residues found after 24, 48, and 72 hr. The values for valine and isoleucine were obtained from 72-hr hydrolysates only.

Half-cystine was determined as cysteic acid (Moore, 1963) after performic acid oxidation of the proteins. The values given are those obtained from a 22-hr hydrolysate. Tryptophan was estimated from the ultraviolet spectrum (Beaven and Holiday, 1952). Duplicate analyses were performed on the protein with the use of 6 M HCl which contained 0.4% phenol to protect the tyrosine.

The activities of both *P. fischeri* and MAV luciferases were assayed by measuring the initial light intensity (I_0) upon mixing rapidly with 1 ml of reduced flavin (5×10^{-5} M) in the presence of aldehyde and oxygen, in a final volume of 2.2 ml. In addition to luciferase, the reaction mixture contained 4×10^{-5} M dodecanal (as an emulsion prepared by ultrasonication), 0.1% bovine serum albumin, and 0.01 M phosphate buffer (pH 6.8). Reactions were carried out at 22 ± 2°.

Light was detected by the use of photomultiplier tubes calibrated with the standard of Hastings and Weber (1963), amplified, and recorded graphically.

Chemical reagents were of analytical quality where available.

Results

Purification and Crystallization of MAV Luciferase. A summary of the purification of MAV luciferase in which the specific activities are estimated using the absorbance at 280 nm is presented in Table II. The cells were grown in a 50-gal batch fermenter with vigorous aeration to a cell density of about 5×10^9 cells/ml, rapidly cooled, and then harvested using a Sharples centrifuge. Cells were lysed osmotically in

TABLE II: Purification of "MAV" Bacterial Luciferase.[a]

Description A	Volume (ml) B	A_{280} C	A_{260} D	$A_{280}:A_{260}$ E	I_0 of $FMNH_2$ (dodecanal) (qsec^{-1} ml^{-1} $\times 10^{-12}$) F	Total Act. (B $\times$ F) (qsec^{-1} $\times$ 10^{-15} G	Sp Act. (F/C) (qsec^{-1} mg^{-1} $\times 10^{-12}$) H
1. Lysate	7640	49	96	0.51	15	115	0.31
2. 45–70% $(NH_4)_2SO_4$ precipitate	757	330	616	0.54	150	114	0.46
3. After RNase, DNase and dialysis	1140	230	400	0.58	51	58	0.22
4. Precipitated fractions after DEAE	64	83	68	1.2	780	50	9.4
5. Precipitated fractions after Sephadex	26.3	83	50	1.7	1620	43	19.3
6. 1st crystallization	6.6	46.5	26.3	1.77	1220	8.05	26.2
7. 2nd crystallization	5.0	36.7	20.5	1.71	1220	6.1	32.5

[a] Summary of results of purification of bacterial luciferase from 1090 g of cells of the MAV strain. Lines 1 through 7 refer to preparations which may be identified by reference to the text. The activity is expressed as the initial maximum light intensity (I_0) which is reached shortly after mixing.

batches of about 200 g by the addition of cold distilled water (600 ml/100 g of cells) to the damp cell paste. After stirring the combined lysates for 1 hr at 4°, followed by a brief (1 min) ultrasonic treatment with a Branson (Model S-75) sonicator, sufficient 2 M phosphate buffer (pH 8.3) was added to bring the buffer concentration to 0.05 M. Solid ammonium sulfate was added to bring the solution to 45% of saturation and the debris and other precipitated material were removed by centrifugation. To the supernatant (line 1), $(NH_4)_2SO_4$ was added to 70% of saturation and the precipitated material (line 2) was dissolved in a standard buffer (0.1 M phosphate, pH 7, with 10^{-3} M EDTA and 10^{-4} M dithiothreitol). The solution was treated with DNase and RNase (Worthington, 10 mg each with 60 mg of $MgSO_4$/l. of the preparation) for 4 hr at 25°, followed by dialysis for 24 hr against standard buffer (line 3).

The protein was then subjected to DEAE chromatography. The most active fractions were pooled, reprecipitated with $(NH_4)_2SO_4$, assayed (line 4), and then applied directly to a Sephadex G-100 column. The active fractions were again pooled, reprecipitated with $(NH_4)_2SO_4$, and assayed (line 5). The luciferase was then crystallized by dialysis against ammonium sulfate, beginning at 35% of saturation and increasing daily by 1% until a sheen appeared, usually at about 40% of saturation. After about 1 week, the crystals which formed were redissolved in the standard buffer, assayed (line 6), and then recrystallized using the same procedure. The crystals obtained are shown in Figure 1. The purity, as judged by acrylamide gel electrophoresis, was good (see below).

Enzymatic Characterization of the Luciferases

Effects of Aldehydes. One of the unusual features of the *in vitro P. fischeri* bacterial luciferase reaction is that light emission persists for a long time after all free substrate ($FMNH_2$) has gone. Analysis has shown that this can be attributed to the presence of an intermediate having a lifetime of many seconds (or indeed tens of seconds), which results from the reaction of luciferase with reduced flavin and oxygen. In effect, the turnover number for luciferase is very small. Although formation of this intermediate is not dependent upon the presence of aldehyde, its lifetime is markedly different when aldehydes of different chain lengths are employed. The quantitative aspects of these effects for the MAV luciferase are strikingly different from those which have been described for *P. fischeri* luciferase (Hastings *et al.*, 1963, 1966).

The straight-chain aliphatic fatty aldehydes with chain lengths from three to fourteen carbon atoms (except tridecanal) were examined in the present study. Even though the

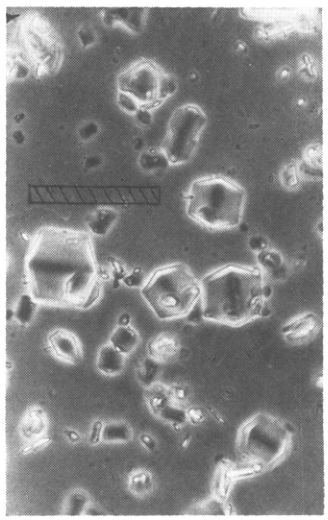

FIGURE 1: Photograph of crystals of the MAV luciferase in 40% $(NH_4)_2SO_4$. Bar scale corresponds to 50 μ.

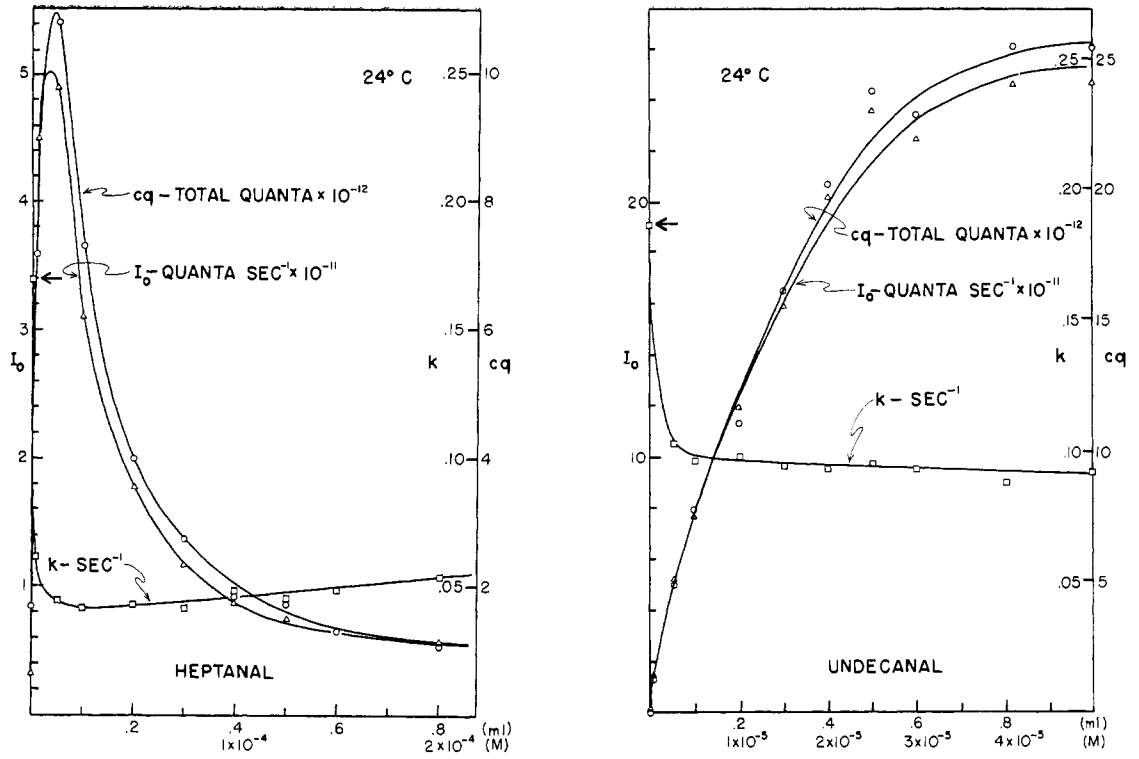

FIGURE 2: The effect of aldehyde concentration upon the initial maximum intensity (I_0), first-order decay constant (k), and relative quantum yield (cq). Ordinates as labeled; abscissa, milliliters of saturated aqueous solution of the aldehyde in a final assay volume of 2.2 ml. The arrows along the ordinates are the values obtained with no added aldehyde. The assay mixture included 0.02 ml of 1.25 M phosphate buffer (pH 6.8), 0.20 ml of 1% bovine serum albumin, 1 μl of luciferase (58.9 mg/ml), and water and saturated aldehyde solution to a volume of 1.2 ml. Reactions were initiated by injecting from a syringe 1.0 ml of $FMNH_2$ (5×10^{-5} M), catalytically reduced by bubbling hydrogen through the FMN solution in the presence of platinized asbestos.

longer chain lengths are only sparingly soluble, the stock solutions may be prepared as saturated aqueous solutions. The concentrations of such solutions were reported previously (Hastings *et al.*, 1963); for example, dodecanal is about 4×10^{-5} M. Excess pure aldehyde (freshly purified by gas chromatography) was mixed with water and centrifuged to remove aldehyde droplets. Varying amounts of such aldehyde solutions (up to 1 ml) were then added to the reaction mixtures which, aside from the variable aldehyde component, were identical with those described in the previous section. Results are presented for two of the aldehydes, heptanal and undecanal (Figure 2). Each point is the average of three determinations. The data from these and similar experiments with the other aldehydes are summarized in Figure 3, which gives the values for the maximum initial intensity, the first-order rate constant for the decay, and the relative quantum yield.

The case of tetradecanal is an exception, since a far greater initial light intensity (up to ten times) can be obtained if excess (*e.g.* sonicated) tetradecanal is added. However, the decay constant (k) increases proportionately so that the relative quantum yield (cq) remains constant. The value plotted (Figure 3) was obtained using 1 ml of a saturated solution of the aldehyde as described above.

Although the general pattern of the aldehyde dependency is similar to that of the *P. fischeri* luciferase, there are several distinctive differences. First, decanal is the optimal aldehyde for initial maximum intensity of the MAV enzyme, whereas tetradecanal was found to be most effective for *P. fischeri* luciferase. As with *P. fischeri*, the values for the initial maximum intensity are closely paralleled by the values for the first-order decay constant, but the absolute values for the latter fall in a somewhat lower range with the MAV luciferase. Bioluminescence with MAV may thus extend over a considerably longer time, *i.e.*, the lifetime of the intermediate is greater (Figure 4). In fact, this was the feature which first attracted our attention and led us to examine the properties of this luciferase. The MAV luciferase might be referred to as "slow" luciferase. However, the remarks above concerning the activity of the luciferase in the presence of sonicated tetradecanal should be noted.

We also observed that high aldehyde concentrations are powerful inhibitors for the MAV but not for the *P. fischeri* luciferase. Although this is evident only for heptanal in Figure 2, it is equally true for other aldehydes, including undecanal and dodecanal, if excess aldehyde is added, *viz.* in the form of a stable emulsion prepared by ultrasonic treatment. The marked stimulation by sonicated tetradecanal is apparently an exception.

The fact that the relative quantum yield is equally high with several different aldehydes is clearly evident (Figure 3). Yields are equally high with all aldehydes from octanal to dodecanal. Since the solubilities of the longer chain-length aldehydes are lower, however, the actual aldehyde concentrations required to give maximum activity are also lower. Thus, longer

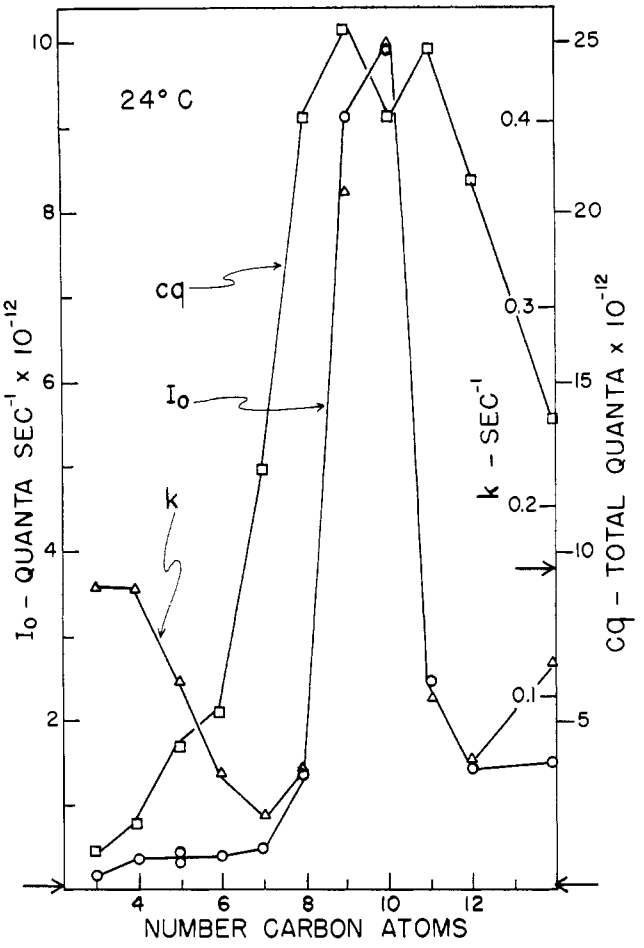

FIGURE 3: Effect of aldehyde chain length on I_0, cq, and k, taken from the several experiments similar to those of Figure 2. Ordinates as indicated; abscissa, the number of carbon atoms in the aliphatic aldehyde used; maximum values of cq and I_0 are presented. The value for k was taken at the aldehyde concentration optimal for I_0. The values for tetradecanal are explained in the text. The arrows along the ordinates are the values obtained with no added aldehyde.

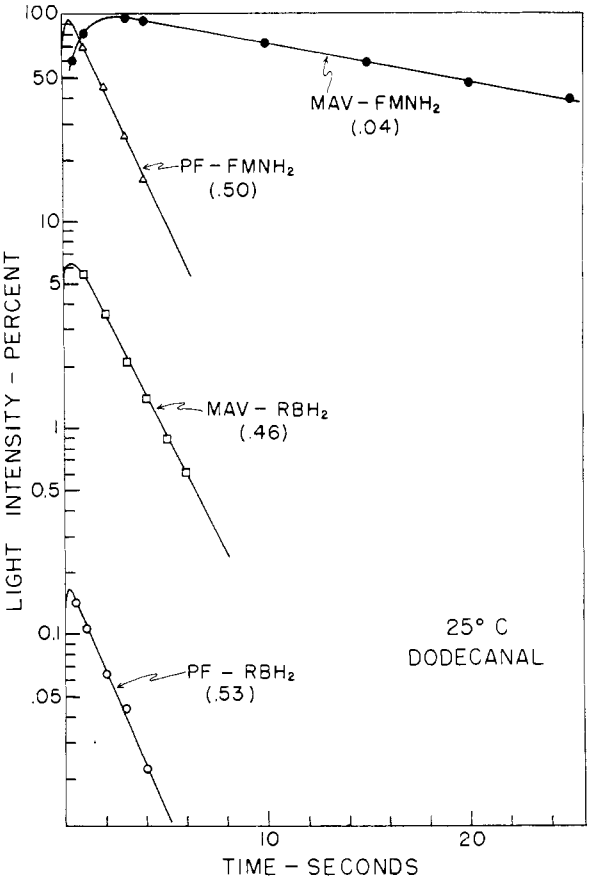

FIGURE 4: Times course of reactions with MAV and PF luciferases, comparing the effects of $FMNH_2$ and reduced riboflavin (RbH_2) upon the intensity and decay rate of the *in vitro* bioluminescence. Assay conditions as specified in Materials and Methods, except that different reduced flavins were used. The values of the first-order rate constants for the decay of luminescence (in sec^{-1}) are noted in parentheses next to the corresponding experiments. Note: PF stands for *P. fischeri*.

chain-length aldehydes are actually far more effective on a molar basis than would be surmised from the plot of Figure 3.

Reactions with Reduced Flavins. When luciferase was first isolated, there were experiments reported (Strehler *et al.*, 1954) which indicated that reduced riboflavin was as effective as reduced riboflavin 5-phosphate ($FMNH_2$) in stimulating luminescence. But in studies with purified extracts the response to riboflavin was found to be far less (McElroy *et al.*, 1954), and it was concluded that the reaction was specific for $FMNH_2$ (Hastings and McElroy, 1955; McElroy and Green, 1955). The riboflavin response was attributed to the presence of FMN in crude extracts which was being reduced by riboflavin.

With the MAV luciferase we were surprised to find a rather good response to reduced riboflavin, and at first attributed it also to endogenous FMN. Upon purification it became clear that this was not the case and that the MAV luciferase itself has activity with reduced riboflavin. The response of the pure *P. fischeri* enzyme was studied again and confirmed to be very weak; but the activity which does occur can also be truly attributed to riboflavin and not to an impurity (Mitchell and Hastings, 1969).

The experiments revealed another and more interesting difference, namely, that the decay time can be influenced by the specific flavin being used (Figure 4). Although the lifetime of the intermediate with the *P. fischeri* luciferase is the same with FMN and riboflavin, this is not so with the MAV luciferase.

The color of the light emitted has also been found to differ slightly with the two luciferases. With $FMNH_2$, for example, the emission peaks were observed to be at 496 and 492 nm with *P. fischeri* and MAV luciferases, respectively. Similarly, small differences were found with other flavins. It is interesting that analogous results have been reported by Seliger and McElroy (1964) in the firefly system. They found quite large differences in the color of the emitted light from different species and attributed this to differences in the luciferases.

Activity vs. pH. The pH–activity profile of an enzyme may be a rather sensitive index for detecting small structural changes in the protein. In the case of the two luciferases there is a really great difference between the two profiles, implying that the structural differences are also substantial.

No study of the effect of pH upon the activity of bacterial luciferase has been reported previously. When luciferase was first isolated, its activity was measured by adding reduced

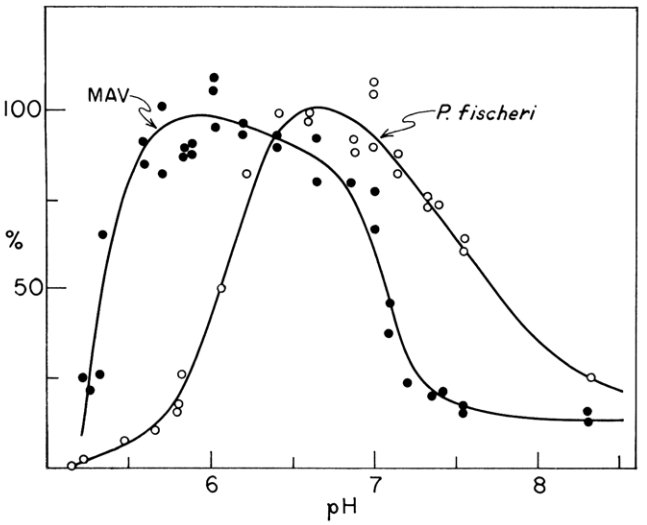

FIGURE 5: Effect of pH upon the activity of the two luciferases. Ordinate, initial maximum light intensity as per cent of the highest value for each enzyme. Reaction mixtures as specified in the text (using dodecanal) except that other phosphate buffers were used to obtain the different pH values.

pyridine nucleotide, which reduces the added FMN *via* a different and distinct enzyme; the pH profiles reported at that time (McElroy *et al.*, 1954), which exhibited a rather sharp optimum at pH 6.8, thus reflected the response of the two enzymes which act in sequence.

The pH profiles determined in our laboratory some years ago using *P. fischeri* luciferase had shown a broad profile, with good and nearly optimal activity over the range pH 6.4–7.2. The use of an assay buffered at about pH 6.8 was thus retained. A determination of the pH profile for the MAV luciferase shows that it is quite different, having its optimum in the region pH 5.6–6.8, and dropping off sharply at higher values. These results, along with a redetermination of the profile for the *P. fischeri* enzyme, are presented in Figure 5.

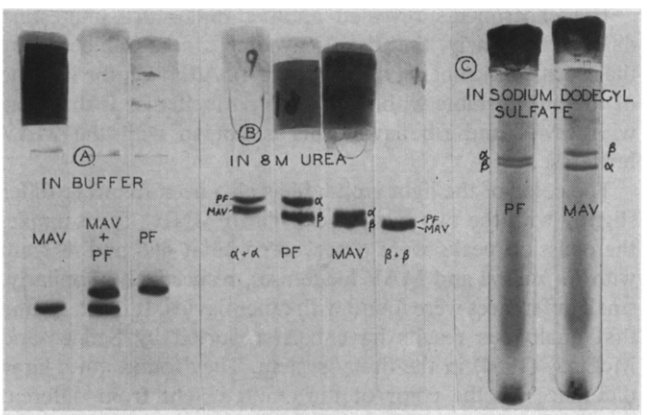

FIGURE 6: Patterns from acrylamide disc gel electrophoresis. (A) Purified preparations of MAV (50γ) and PF (45γ) luciferases and the coelectrophoresis of a mixture of the two, in Tris–tricine buffer (pH 8.5). (B) Purified PF (91γ) and MAV (60γ) luciferases and the coelectrophoresis of mixtures of the subunit pairs (30γ each) from the two luciferases (isolated as shown in Figure 7) in 8 M urea at pH 8.7. (C) Purified PF (10γ) and MAV (12γ) in sodium dodecyl sulfate in phosphate buffer (pH 7.2) showing subunit structure and distinctive differences in molecular weights of all subunits. Note: PF stands for *P. fischeri*.

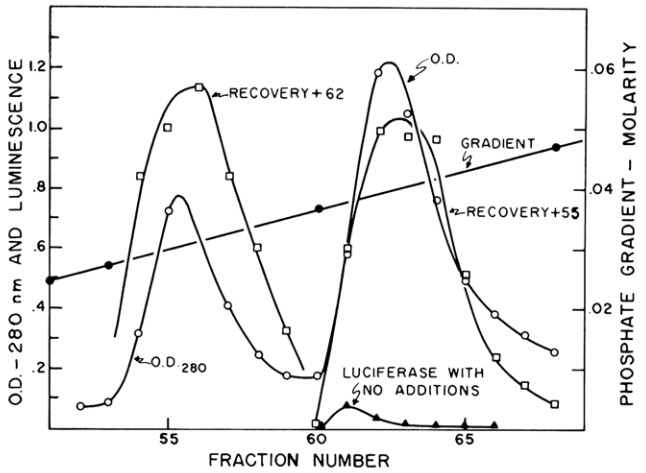

FIGURE 7: Separation of MAV luciferase subunits by DEAE-cellulose chromatography in the presence of 8 M urea eluted with a linear phosphate gradient at pH 7.0. Protein was estimated by absorbance at 280 nm. Recovery of luciferase activity was measured 14 hr after dilution into buffer to lower the urea concentration. Assay mixture for luciferase described in text. The recovery of luciferase activity occurs in the region of the α subunit if some β is added (□) and in the region of β if some α is added. With no additions some recovery occurs in the region where some of both subunits is present in the tubes (▲).

A matter of considerable relevance with regard to mechanism relates to the apparent first-order decay constants, which are relatively invariant for the bioluminescence at the different pH values. A practical consequence is that a plot of the quantum yield as a function of pH closely parallels the curve of Figure 5. The pH thus seems to affect the formation of the long-lived intermediate, rather than its lifetime.

Physical Characterization of the Luciferases

Disc Electrophoresis of the Proteins and Their Subunits. Acrylamide disc electrophoresis was carried out using highly purified luciferases of the two types. Individually each protein migrated as a single band as determined both by staining (Figure 6A) and by luciferase activity. The R_F values of the *P. fischeri* and MAV luciferases were 0.7 and 0.8, respectively. Similarly, a good separation of a mixture of the two luciferases occurred upon coelectrophoresis on a single gel; two separated peaks of activity could be identified with the stainable bands, and these corresponded to the MAV and *P. fischeri* types of activity (Figure 6A).

Structural studies with *P. fischeri* luciferase had shown that it is comprised of nonidentical subunits, differing in charge but identical or nearly so in molecular weight (Friedland and Hastings, 1967b).

Acrylamide gel electrophoresis of the MAV luciferase in the presence of 8 M urea showed two major bands of equal intensity, presumed to represent two nonidentical subunits similar to those found with *P. fischeri* luciferase. They were thus also designated α and β by their relative charge, as determined by banding position on the gel, the β being the more negatively charged polypeptide chain. The R_F values for the α subunits from the two luciferases differed considerably, but the two β subunits migrated with about the same R_F

TABLE III: Molecular Weights of the Two Luciferases Determined by Acrylamide Gel Electrophoresis in Sodium Dodecyl Sulfate.

	P. fischeri	MAV
α	41,000	37,000
β	38,000	42,000

value (Figure 6B). It thus appeared possible that the two β subunits might be identical.

Subunit Isolation and Molecular Weight Determination. The presence of two different subunits in each protein was firmly established by their separation on DEAE-cellulose in the presence of 8 M urea (Figure 7). This technique provides subunits in milligram quantities; using acrylamide gel electrophoresis it was possible to show that the subunit which eluted first, at a lower ionic strength, was the α subunit.

The molecular weight of these subunits was estimated using electrophoresis in sodium dodecyl sulfate–polyacrylamide gels, a technique which separates polypeptide chains according to their molecular weight (Weber and Osborn, 1969). By this procedure all four subunits were found to be clearly different in molecular weight (Figure 6C).

The identity of the bands was established by using isolated subunits of both *P. fischeri* and MAV separated as shown in Figure 7. From this it was found that in the *P. fischeri* luciferase the α subunit is the heavier, while in the MAV luciferase the β is heavier (Table III). Although the absolute molecular weights are considered accurate only to within 10%, the relative mobilities of the subunits on the sodium dodecyl sulfate gels are highly reproducible. The relative molecular weights of the subunits are thus established as MAV β > *P. fischeri* α > *P. fischeri* β > MAV α.

Since the α and β bands are of nearly equal intensity, a 1:1 ratio of the two subunits is inferred for both luciferases, giving a molecular weight of 79,000, in good agreement with the value of 76,000 calculated from the diffusion coefficient measured during sedimentation velocity runs (Hastings *et al.*, 1965).

Hybridization Attempts. In previous studies with the *P. fischeri* luciferase it had been found that neither the α nor the β subunit possesses activity alone after renaturation. A small residual activity could always be found and was attributed to incomplete separation. In view of the similar (though not identical) properties of the subunits from the two luciferases, it seemed possible that hybrid luciferases might be formed by renaturation of the correct pairs.

To test this possibility the subunits of MAV luciferase were isolated by chromatography on DEAE-cellulose in the presence of 8 M urea (Figure 7). A similar isolation of the subunits from *P. fischeri* luciferase provided samples of those subunits, so that the subunits could be mixed in all combinations and studied with regard to the recovery of activity. The existence and amount of contaminating partner is evident by the activity recovered from individual tubes (see Figure 7 and Table IV). It would have been readily possible to detect the presence of an active hybrid molecule by the altered kinetics of the decay or by the increased luciferase activity. No stimulation of activity occurred in any combination (Table IV). Figure 8 illustrates the results of these experiments, which clearly show that the two β subunits are different in that they do not react with the α of the other proteins to give α-type kinetics. A similar conclusion was arrived at with regard to the other combinations.

TABLE IV: Hybridization Attempts between Subunits from Different Luciferases.[a]

Subunit Present During Renaturation	Activity (I_0) Obtained
P. fischeri α (2.23)	0.3
P. fischeri β (1.12)	45
MAV α (1.27)	0.01
MAV β (2.37)	4.5
P. fischeri α + *P. fischeri* β	880
MAV α + MAV β	120
P. fischeri α + MAV α	0.5
P. fischeri α + MAV β	3.5
P. fischeri β + MAV α	34
P. fischeri β + MAV β	39

[a] Activities of luciferase subunits combined in different combinations and allowed to renature. The subunits from the two different luciferases were isolated using chromatography on DEAE in 8 M urea; the absorbances at 280 nm of the four samples in 8 M urea prior to dilution are noted in parentheses. A 0.01 volume of each subunit sample was added to 1 ml of the buffer (0.25 M phosphate, pH 7 with 10^{-3} M dithiothreitol and 0.2% bovine serum albumin). At 4° the recovery of luciferase activity was determined with the assay mixture specified under Materials and Methods using dodecanal. The lower activity of the MAV luciferase is due to the fact that its specific activity with dodecanal is considerably less than that of the *P. fischeri* luciferase with dodecanal.

Amino Acid Composition. Although it appeared reasonable to infer that homology exists, no positive cross-reactions occurred following renaturation of the subunit pairs in any combination (Table IV). Amino acid analyses of the luciferases and their individual subunits were therefore performed in order to see how different the two proteins are. For an easy comparison of the amino acid compositions the results for both the subunits and the native enzymes are presented in Table I on the basis of the number of moles of amino acid residues per 40,000 g.

The fact that the absorbance of the two subunits at 280 nm is quite different may be accounted for by the twofold differences in tryptophan; the subunit having the higher content is α in *P. fischeri* (Friedland and Hastings, 1967b) and β in MAV (Figure 7). In addition, a comparison of the composition of the native enzymes with their subunits shows that the number of residues for any given amino acid in the total enzyme is very close to the average of its two subunits. These results confirm the assumption that the subunits occur in a 1:1 ratio.

With regard to over-all composition, the two intact luciferases differ most with regard to threonine, alanine, and iso-

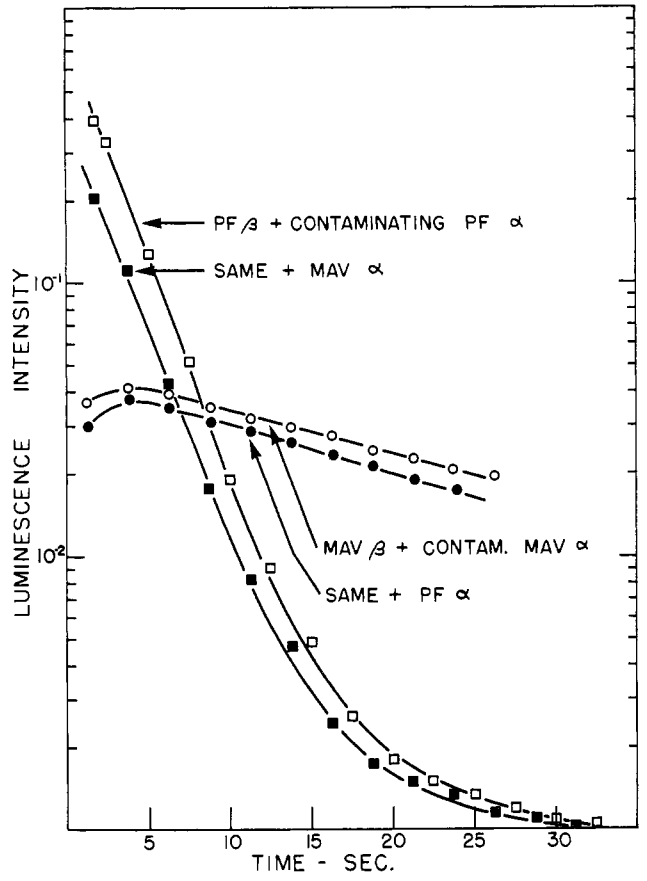

FIGURE 8: Bioluminescent reactions of renatured luciferases. Subunits from the two different luciferases were combined as indicated, diluted into buffer, and allowed to recover. Individual members recover a small amount of activity without addition because of a small contaminant of the normal partner. This activity constitutes the background. Note: PF stands for *P. fischeri*.

leucine. But all four chains are different and distinctive with regard to amino acid composition. The *P. fischeri* α and β exhibit drastic differences in lysine, arginine, and tryptophan and less (but significant) differences in threonine, glycine, valine, and leucine. The MAV α and MAV β are even more distinct; there are substantial differences with regard to at least eight amino acids.

Discussion

This study has served to clarify certain structural features of the bacterial luciferases. From previous studies we were not certain whether luciferase is comprised of four or two subunits. Based on the molecular weight determination of the subunits using sodium dodecyl sulfate gels, it now appears certain that the intact luciferase has a molecular weight close to 80,000 and comprises only two subunits of about 40,000 each. This coincides exactly with our earlier estimate of subunit size based on calibrated Sephadex gel filtration studies both in 5 M guanidine and 8 M urea–formate (Friedland and Hastings, 1967a). The earlier determination of 76,000 for the molecular weight of *P. fischeri* luciferase also is in close agreement (Hastings *et al.*, 1965).

It has also been shown here that the two luciferase subunits (α and β) are distinctively different not only in charge, but also in molecular weight. The differences are greater for the MAV luciferase than for the *P. fischeri* enzyme, explaining the fact that these differences had not been noted in earlier gel filtration studies of the subunits of *P. fischeri* (Friedland and Hastings, 1967a).

The failure of the subunits to cross-react and form a hybrid luciferase was somewhat unexpected in view of their size and charge similarities. The subunits, when renatured individually, are inactive for light emission. In previous work with *P. fischeri* it had been noted that during their renaturation β subunits (but not α) form dimers. This phenomenon stands in the way of achieving 100% recovery of activity from denatured luciferase because the formation of β-β dimers is competitive with the formation of active (α-β) luciferase. It was interesting to note that in the case of the MAV luciferase it is the α subunit which apparently forms such dimers. This suggests that if homology exists among the subunits in the two luciferases, the *P. fischeri* α is homologous with the MAV β, and the *P. fischeri* β with the MAV α. This same possibility was suggested by the molecular weights.

With regard to the broader questions which relate to the matter of homology, it certainly seems likely that the two luciferases are related. They exhibit both structural and enzymatic similarities, and are synthesized by microscopically similar and perhaps closely related bacteria. Yet the enzymes are so different that virtually every property so far examined is quantitatively different. In addition to those reported in this paper (response to substrates, pH–activity profile), the antigenic cross-reactions between the two proteins appear to be very weak or completely absent (K. Nealson, 1969, personal communication). Taken together with the evidence concerning the size, charge, and composition differences of the proteins and their subunits, these results imply that the differences in amino acid sequence and the consequent tertiary structure are substantial, indeed drastic. In spite of these enormous differences there is reason to believe that the luciferases are related, and that the differences represent evolutionary divergence. They both utilize the same substrates, and aldehyde causes an increase in the quantum yield of the reaction. The proteins are very similar in molecular weight and have subunits similar in number and size. The comparison of these two forms may thus be expected to be highly diagnostic with regard to their common structural features associated with the energy conversion and light-emitting step. The similarities between the two luciferases suggest that their active sites may be identical or at least very similar; the large differences elsewhere in these proteins may make it possible to pinpoint the active sites. The amino acid sequence and crystallographic structure determinations should be of special value in this regard.

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References

Beaven, G. H., and Holiday, E. R. (1952), *Advan. Protein Chem.* 7, 319.

Coffey, J. J. (1967), *J. Bacteriol. 94*, 1638.
Cormier, M. J., and Strehler, B. L. (1954), *J. Cell Comp. Physiol. 44*, 277.
Friedland, J. M. (1968), Ph.D. Thesis, University of Illinois, Urbana, Ill.
Friedland, J. M., and Hastings, J. W. (1967a), *Proc. Natl. Acad. Sci. U. S. 58*, 2336.
Friedland, J. M., and Hastings, J. W. (1967b), *Biochemistry 6*, 2893.
Hastings, J. W., and Gibson, Q. H. (1963), *J. Biol. Chem. 238*, 2537.
Hastings, J. W., Gibson, Q. H., Friedland, J., and Spudich, J. (1966), *in* Bioluminescence in Progress, Johnson, F. H., and Haneda, Y., Ed., Princeton, N. J., Princeton University, pp 151–186.
Hastings, J. W., and McElroy, W. D. (1955), *in* The Luminescence of Biological Systems, Johnson, F. H., Ed., Washington, D. C., A.A.A.S., pp 257–264.
Hastings, J. W., Riley, W. H., and Massa, J. (1965), *J. Biol. Chem. 240*, 1473.
Hastings, J. W., Spudich, J. A., and Malnic, G. (1963), *J. Biol. Chem. 238*, 3100.
Hastings, J. W., and Weber, G. (1963), *J. Opt. Soc. Am. 53*, 1410.
Keynan, A., Veeder, C., and Hastings, J. W. (1963), *Biol. Bull. 125*, 382.
McCloskey, R. R. (1960), B. S. Thesis, University of Illinois, Urbana, Ill.
McElroy, W. D., and Green, A. A. (1955), *Arch. Biochem. Biophys. 56*, 240.
McElroy, W. D., Hastings, J. W., Sonnenfeld, V., and Coulombre, J. (1954), *J. Bacteriol. 67*, 402.
Mitchell, G., and Hastings, J. W. (1969), *J. Biol. Chem. 244*, 2572.
Moore, S. (1963), *J. Biol. Chem. 238*, 235.
Seliger, H. H., and McElroy, W. D. (1964), *Proc. Natl. Acad. Sci. U. S. 52*, 75.
Seliger, H. H., and McElroy, W. D. (1965), Light: Physical and Biological Action, New York, N. Y., Academic.
Shapiro, A. L., Vinuela, E., and Maizel, J. V. (1967), *Biochem. Biophys. Res. Commun. 28*, 815.
Spackman, D. H., Stein, W. H., and Moore, S. (1958), *Anal. Chem. 30*, 1190.
Spruit, C. J. P., and Van der Burg, A. (1960), *Biochim. Biophys. Acta 5*, 175.
Strehler, B. L., Harvey, E. N., Chang, J. J., and Cormier, M. J. (1954), *Proc. Natl. Acad. Sci. U. S. 40*, 10.
Weber, K., and Osborn, M. (1969), *J. Biol. Chem. 244*, 4406.