Hybridization of Bacterial Luciferase with a Variant Produced by Chemical Modification*

E. A. Meighen†, M. Ziegler Nicoli,‡ and J. W. Hastings§

ABSTRACT: Hybridization of native and chemically modified oligomeric proteins is a convenient and generally applicable method for the study of subunit structure, function, and interactions. The present work was initiated to confirm the quaternary structure of bacterial luciferase and to elucidate whether functional differences existed between the nonidentical subunits. Bacterial luciferase ($\alpha\beta$) was succinylated with the aim of producing a homogeneous electrophoretic variant, ($\alpha_s\beta_s$), having the same subunit structure as the native enzyme. A suitable, inactive derivative was obtained in which approximately 45% of the 35 lysyl residues of luciferase was succinylated. Hybridization between $\alpha\beta$ and $\alpha_s\beta_s$ was accomplished by dissociation in urea and removal of the denaturant by dilution and/or dialysis. Three components were resolved by electrophoresis and isolated by DEAE-Sephadex chromatography: $\alpha\beta$, the two hybrid species ($\alpha\beta_s$ and $\alpha_s\beta$ in a single band) and $\alpha_s\beta_s$, thus confirming a dimeric structure for luciferase. Reconstitution experiments were conducted between isolated native subunits (α or β) and inactive $\alpha_s\beta_s$ in order to obtain samples containing only one of the hybrid species. The native subunits, isolated by DEAE-Sephadex chromatography in 5 M urea at 4°, gave upon recombination, luciferase with a specific activity of up to 90% of that of the untreated enzyme. Consequently, quantitative complementation of a fixed amount of the α or β subunit could be conducted with variable amounts of $\alpha_s\beta_s$ in order to deduce the specific activities of $\alpha\beta_s$ and $\alpha_s\beta$. These experiments showed that $\alpha\beta_s$ had at least half the specific activity of the native enzyme whereas $\alpha_s\beta$ was virtually inactive. These results, which were confirmed by the isolation and characterization of the individual hybrid species, indicate that the α and β subunits of luciferase have different rather than homologous functions, and provide the basis for future identification of those functions.

Bacterial luciferase is a protein isolated from bioluminescent bacteria which has the unusual capability of catalyzing the conversion of chemical to light energy (Hastings, 1968). The emission of light *in vitro* has been shown to be the result of the bioluminescent oxidation of reduced flavin mononucleotide ($FMNH_2$)[1] by molecular oxygen, involving as well a long-chain aldehyde (Hastings and Gibson, 1963). The mechanism of the reaction, however, is still poorly understood. Among the major unresolved questions are the function of the long-chain aldehyde, the energy source for light emission, and the identity of the light-emitting species.

The enzyme has been isolated from two different strains of bacteria and in both cases consists of two nonidentical subunits (Hastings *et al.*, 1969; Meighen *et al.*, 1970b). The subunits (α and β) can be separated by chromatography in urea (Friedland and Hastings, 1967; Gunsalus-Miguel *et al.*, 1972);[2] upon removal of urea the subunits will recombine to form active luciferase. The function of the different subunits in the bioluminescent reaction is unknown. Separated subunits are themselves functionally inactive after removal of the denaturant, possibly indicating that the functions of these subunits can only be expressed within the constraints of the quaternary structure. The present experiments are concerned with this question, the functions of the α and β subunits being studied with the quaternary structure of luciferase intact.

An electrophoretic variant of an oligomeric protein suitable for hybridization with the unmodified protein can be obtained by limited reaction of the native enzyme with succinic anhydride (Meighen and Schachman, 1970a,b; Meighen *et al.*, 1970a). The moderate succinylation of luciferase results in an inactive derivative designated as $\alpha_s\beta_s$. Hybridization of native and succinylated luciferase should result in the production of two hybrid species, $\alpha\beta_s$ and $\alpha_s\beta$, if, as previously reported (Hastings *et al.*, 1969), luciferase is a heteropolymeric protein containing two subunits. If the α and β subunits are homologous and have the same functional capabilities then the specific activities of $\alpha\beta_s$ and $\alpha_s\beta$ should be the same, possibly 50% of that for $\alpha\beta$. However, if the subunits have different functions, and since $\alpha_s\beta_s$ is inactive, then either one or both of the hybrid species is expected to be inactive, depending upon whether succinylation has inactivated the function of one or both of the subunits. The study of the catalytic activity of the hybrid species thus allows a distinction to be made between subunits that are homologous and have the same function and subunits that have different functions in the bioluminescent reaction. Furthermore, if the subunits have different functions it may be possible to deduce their specific roles in the bioluminescent reaction by investigation of the catalytic properties of the hybrid species, $\alpha\beta_s$ and $\alpha_s\beta$. Such studies should provide valuable information regarding the mechanism of the reaction.

The present paper describes the production of succinylated luciferase and the isolation and characterization of hybrid

* From the Biological Laboratories, Cambridge, and Department of Biological Chemistry, Harvard University, Boston, Massachusetts. *Received May 12, 1971.* This research was supported in part by a National Science Foundation research grant (GB 16512) and by a U. S. Public Health Service training grant (GM 00451). A preliminary report has been published (Ziegler *et al.*, 1971).

† Present address: Department of Biochemistry, McGill University, Montreal, Quebec, Canada.

‡ Predoctoral trainee, USPHS, Department of Biological Chemistry, Harvard Medical School, Boston, Mass. 02115.

§ To whom to address correspondence at The Biological Laboratories, Harvard University, Cambridge, Mass. 02138.

[1] Abbreviations used are: $FMNH_2$ and FMN, reduced and oxidized riboflavin 5′-phosphate; BSA, bovine serum albumin; DTT, dithiothreitol; GPDH, glyceraldehyde-3-phosphate dehydrogenase; ATCase, aspartate transcarbamylase.

[2] Manuscript in preparation.

species containing one native and one succinylated luciferase subunit. The experiments confirm previous evidence showing that luciferase is a dimer, and indicate that the nonidentical subunits have different functions in the bioluminescent reaction. The subsequent paper investigates functional and structural properties of the hybrid species, $\alpha\beta_s$ and $\alpha_s\beta$, and compares them with $\alpha\beta$ and $\alpha_s\beta_s$.

Experimental Section

Materials. Luciferase was isolated from *Photobacterium fischeri*, strain MAV, and purified according to the procedure of Gunsalus-Miguel *et al.* (1972).[2] The purified protein had a specific activity of 1000 light units (LU)/mg at 22°, where 1 LU is equivalent to 2.2×10^{10} quanta/sec. Its concentration was determined spectrophotometrically on the basis of a specific absorbance coefficient of 0.94 (0.1%, 1 cm) at 280 nm. A molecular weight of 7.9×10^4 was used for luciferase in all calculations (Hastings *et al.*, 1969). The α and β subunits were separated by DEAE-Sephadex chromatography in 5 M urea (4°) and stored in urea at −20° (Gunsalus-Miguel *et al.*, 1972).[2]

Urea (Merck) solutions were prepared immediately prior to use, deionized with a mixed-bed resin (Amberlite MB-1), treated with activated charcoal, and then Millipore filtered. In some cases, Ultra Pure urea (Mann) was used without further treatment.

Phosphate buffers were made by mixing appropriate volumes of K_2HPO_4 and NaH_2PO_4 solutions. DEAE-Sephadex A-50 was obtained from Pharmacia.

Luciferase Assay. One milliliter of 5×10^{-5} M $FMNH_2$ (prepared by bubbling H_2 through a solution of FMN [Sigma] containing platinized asbestos) was injected into 1.2 ml of 0.02 M phosphate (pH 7.0) containing 2 mg of BSA (Pentex, crystallized), 0.01 ml of a 0.1% (v/v) sonicated suspension of aldehyde (Aldrich), and enzyme. The aldehyde (dodecanal unless otherwise specified) was added to the assay solution about 60sec prior to the injection of $FMNH_2$. The light emission was detected by the use of a photometer (Mitchell and Hastings, 1971), calibrated with the standard of Hastings and Weber (1963).

Succinylation of Luciferase. Succinylated luciferase was prepared by addition of solid succinic anhydride (Eastman Organic Chemicals) to a 1% solution of luciferase in 0.05 M Tris-chloride (pH 8.0) at room temperature. The pH was maintained at 8.0 by the addition of 1.0 N NaOH until the completion of the reaction (about 20 min). The succinylated luciferase was then dialyzed exhaustively at 4° against the desired buffer. The amount of succinic anhydride added per mole of luciferase was based on the total number of lysyl residues, namely, 35/molecule of luciferase (Hastings *et al.*, 1969). The number of free amino groups was estimated by the method of Moore and Stein (1948) as modified by Fraenkel-Conrat (1957).

Sedimentation Studies. Sedimentation velocity experiments were performed with a Spinco Model E ultracentrifuge using the schlieren optical system. Double-sector cells with a 12-mm optical path, quartz windows, and aluminum-filled, epoxy centerpieces were used. Experiments were conducted at a constant temperature at a rotor speed of 52,000 rpm. Based on the amino acid composition (Hastings *et al.*, 1969), a partial specific volume of 0.724 ml/g was calculated (Cohn and Edsall, 1943). Photographic plates (Metallographic) were analyzed with the aid of a Gaertner microcomparator. The area of the boundary for succinylated luciferase sedimenting at a rate similar to native luciferase was integrated. This was compared to the area observed for native luciferase of known concentration at the same radial position in the cell and the same phase-plate angle. In this way, the concentration of succinylated molecules with the quaternary structure of native luciferase could be estimated.

Zone Electrophoresis. Electrophoresis was conducted on gelatinized cellulose polyacetate strips (Colab Laboratories) in 0.04 M potassium phosphate–0.001 M EDTA (pH 7.0). A voltage of 200 V was applied for 20 min to 14.6-cm strips in a Microzone electrophoresis cell (Beckman-Spinco) or 45 min to 20 cm strips in a Colab electrophoresis apparatus.

The protein was fixed and stained by immersion of the membrane in a solution of ponceau S in trichloroacetic acid and sulfosalicylic acid (Beckman-Spinco) for approximately 10 min. The membrane was rinsed in 5% acetic acid, immersed in 0.002% nigrosin in 2% acetic acid for several hours, and either stored in 5% acetic acid or dehydrated and dried by immersing for 1 min in 37% formaldehyde and then 3 min in 7% glycerol followed by drying at 80–90°.

Hybridization of Native and Succinylated Luciferase. Experiments were conducted with native luciferase to determine optimal conditions (at 4°) for recovering milligram quantities of active enzyme after dissociation into subunits in 4 M urea. Maximum luciferase activity was recovered 24–48 hr after removal of the urea. This was done by dialysis and/or dilution into 0.04 M phosphate–0.001 M EDTA (pH 7.0) containing DTT. The maximum recovery of activity (about 60%) was obtained if the protein concentration in the reconstitution mixture was 0.1–0.4 mg/ml. Native and succinylated luciferase were hybridized by this procedure. Samples were incubated at concentrations of 1–2 mg/ml in 4 M urea–0.04 M phosphate–0.001 M EDTA–0.05 M DTT for 30 min at 4°, diluted 1:5 into 0.04 M phosphate–0.001 M EDTA (pH 7.0), and dialyzed exhaustively for 40 hours against the same buffer containing 2×10^{-4} M DTT. The resulting mixtures were concentrated by placing the dialysis bags in solid Aquacide II (Calbiochem) at 4° and then dialyzed against the desired buffer.

In some experiments, in which it was only important to recover activity and not to isolate or characterize the reconstituted proteins, no dialysis or concentration steps were used and BSA was included in the recovery buffer. The samples were simply diluted (1:100) into 0.1 M phosphate–0.001 M EDTA–0.01 M DTT–0.2% BSA (pH 7.0) at 4°; activity was measured after 40 hr. Higher yields of activity (up to 90%) were obtained by this procedure, attributable, we believe to the presence of BSA.

Results

Chemical Modification of Luciferase with Succinic Anhydride. Bacterial luciferase was reacted with limited amounts of succinic anhydride in order to obtain a variant suitable for hybridization with the native enzyme. Such a variant must not only be electrophoretically distinct from the unmodified protein but must possess the same quaternary structure and be sufficiently homogeneous so that members of the hybrid set can readily be separated (Meighen and Schachman, 1970a). Consequently, a number of different preparations of succinylated luciferase were compared with native luciferase with respect to sedimentation properties, electrophoretic mobility, activity, and homogeneity.

The addition of succinic anhydride to bacterial luciferase results in the introduction of negatively charged succinyl groups onto the positively charged lysyl residues (Table I).

TABLE I: Properties of Succinylated Luciferase.

Sample	Moles of Succinic Anhydride Added/Lysyl Residue	Percentage of Lysyl Residues Succinylated[a]	$s_{20,w}$[b] (S)	Percentage of Intact Molecules[c]
A	0	0	4.8	100
B	2.5	29	4.9	82
C	4.5	46	5.3	58
D	5.0	49	5.3	45

[a] The fraction of lysyl residues succinylated was estimated by ninhydrin analysis as described in the Experimental Section. [b] Sedimentation velocity experiments were performed in solutions containing 0.5 M NaCl–0.04 M phosphate–0.001 M EDTA–10^{-4} M DTT (pH 7.0) at 26°. The initial protein concentrations were approximately 8 mg/ml. Additional details are given in the Experimental Section. [c] The concentration of intact molecules was estimated from the area corresponding to the sedimenting boundary (*e.g.*, see Figure 1) and divided by the total protein concentration.

The two nonidentical subunits, α and β, have molecular weights of 4.2×10^4 and 3.7×10^4, and possess 20 and 15 lysyl residues, respectively (Hastings *et al.*, 1969). Based on these numbers, and assuming similar reactivities for the lysyl residues of the different subunits, about 10 and 8 residues would be modified in α and β, respectively, in samples such as D (Table I). The succinylation of other residues in luciferase (*e.g.*, sulfhydryl) was not investigated.

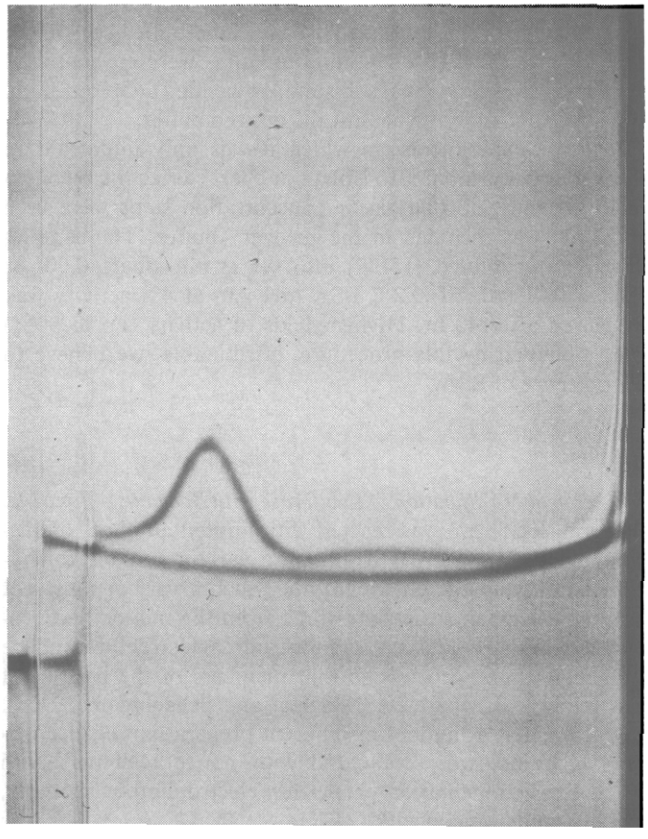

FIGURE 1: Sedimentation velocity pattern of succinylated luciferase (sample C, Table I). Movement is from left to right. The photograph was taken with a phase-plate angle of 60°.

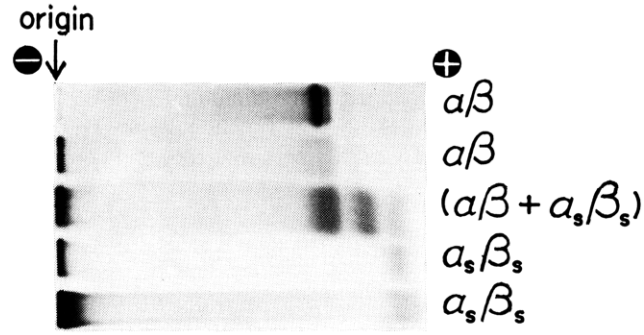

FIGURE 2: Electrophoresis on cellulose acetate of the hybrid set of native ($\alpha\beta$) and succinylated ($\alpha_s\beta_s$) luciferases. Succinylated luciferase was prepared by reaction of luciferase with 5.0 moles of succinic anhydride per lysyl residue. As described in the Experimental Section, native and succinylated luciferase were hybridized by dissociation and reconstitution. Electrophoresis was conducted for 12 min at 200 V and the membrane stained for protein. Variations in staining intensity are primarily due to differences in protein concentration in the samples. The mobility of native luciferase was found to be -1.8×10^{-4} cm²/(V sec). Samples 2, 3, and 4 were dissociated and reconstituted prior to electrophoresis.

Table I gives the values obtained in one set of experiments for the sedimentation coefficient and percentage of intact molecules as a function of the extent of modification of the lysyl residues of luciferase. Irrespective of the extent of succinylation only one peak was observed, sedimenting at the same rate as native luciferase (Figure 1). The small increase in sedimentation coefficient for samples C and D over that for native luciferase cannot be readily accounted for but may reflect a dependence on the protein concentration. Some aggregated protein is also present in the succinylated luciferase samples (Figure 1). The relative quantity of intact molecules decreased as a function of the extent of succinylation. This was calculated by comparing the area under the sedimenting peak with the area observed for native luciferase in which no aggregated material was present. No species sedimenting at a rate slower than native luciferase were observed in any experiment.

Electrophoresis experiments were conducted on the different succinylated preparations. Each of the succinylated samples contained only one migrating component with a negative mobility greater than that of native luciferase (*e.g.*, $\alpha_s\beta_s$, Figure 2). In addition, material was present at the origin. As the extent of succinylation increased, the relative amounts of the protein at the origin increased and the quantity of the single migrating component decreased. This result, in conjunction with the sedimentation velocity experiments, shows that only the succinylated material with the same quaternary structure as luciferase migrated upon electrophoresis.

Although sample B (Table I) had an electrophoretic mobility greater than native luciferase, it was too heterogeneous to be suitable for hybridization experiments, as judged by the width of its electrophoretic band. Samples C and D had mobilities 16 and 22% greater than native luciferase and were sufficiently homogeneous for hybridization experiments with the native enzyme. Both were relatively inactive; sample C had 2% and D had 0.8% the specific activity of the native enzyme, based on the concentration of intact succinylated molecules. With other similarly succinylated preparations of luciferase specific activities ranged from 0.2 to 5%.

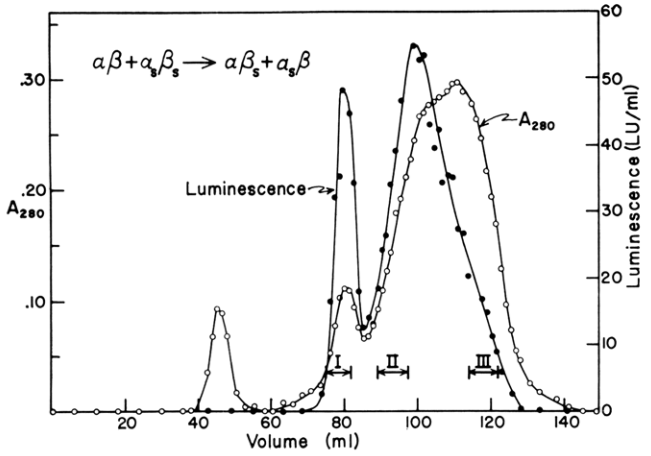

FIGURE 3: DEAE-Sephadex chromatography of a hybrid set of native ($\alpha\beta$) and succinylated ($\alpha_s\beta_s$) luciferases. Succinylated luciferase was prepared by reaction of 4.5 moles of succinic anhydride/lysyl residue with native luciferase. Native and succinylated luciferase were mixed in urea at a molar ratio of 1:4 and hybridized by treatment with 4 M urea. The hybrid mixture (3.5 ml) was applied to a 1 × 50 cm column of DEAE-Sephadex preequilibrated in 0.001 M EDTA–0.2 M NaCl–0.05 M Tris-chloride (pH 7.5). The column was eluted (10 ml/hr) with a linear gradient of 100 ml of the equilibration buffer *vs.* 100 ml of the same buffer made 1.1 M in NaCl. The fractions were collected and analyzed for absorbance at 280 nm (A_{280}, ○) and luminescence activity (LU/ml; ●) and then pooled as indicated (I, II, and III). The abscissa represents the gradient volume eluted from the column (total milliliters of eluent minus the dead volume of the column).

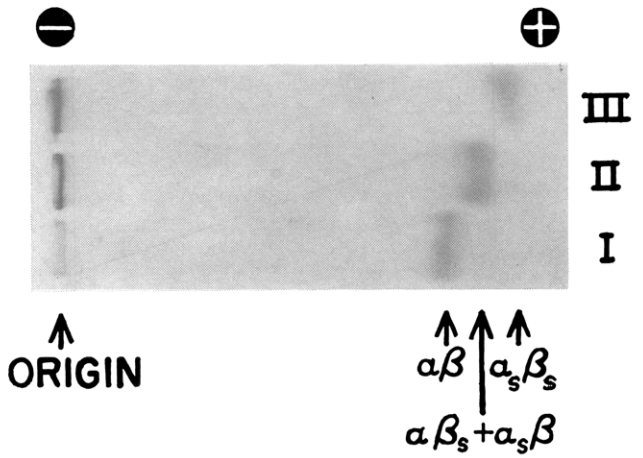

FIGURE 4: Cellogel electrophoresis of the components of the hybrid set of native and succinylated luciferase (I, II, and III) isolated by DEAE-Sephadex chromatography (see Figure 3). Electrophoresis was conducted at 200 V for 45 min and the membrane stained for protein as indicated in the Experimental Section. The positions of native ($\alpha\beta$) and succinylated luciferase ($\alpha_s\beta_s$) as well as the hybrid species ($\alpha\beta_s$ and $\alpha_s\beta$) were established in other experiments in which I or III were electrophoresed with authentic $\alpha\beta$ or $\alpha_s\beta_s$, respectively.

Since qualitative detection of the members of the hybrid set[3] was important, sample D was judged more suitable for hybridization experiments because it was more homogeneous than sample C upon electrophoresis. Furthermore, because of its lower enzyme activity, this sample was more suitable for complementation with the isolated α or β subunits. However, for hybridization experiments in which it was desired to isolate members of the hybrid set in quantity (*e.g.*, by DEAE-Sephadex chromatography), sample C was more suitable since it had a higher percentage of intact succinylated luciferase molecules.

Hybridization of Native and Succinylated Luciferase. Figure 2 shows the electrophoretic pattern after hybridization of native and succinylated luciferase. Native luciferase migrates toward the anode with its characteristic mobility (-1.8×10^{-4} cm^2/(V sec)), while succinylated luciferase ($\alpha_s\beta_s$) exhibits a mobility of -2.2×10^{-4} cm^2/(V sec). The electrophoretic patterns of $\alpha\beta$ and of $\alpha_s\beta_s$ after dissociation and reconstitution show that these molecules have refolded after dissociation in urea, and that no additional bands have been produced. However, some material remains at the origin, presumably due to incomplete reconstitution of the subunits as well as the presence of aggregated succinylated material.

The hybridized sample ($\alpha\beta$ and $\alpha_s\beta_s$) exhibits bands not only for $\alpha\beta$ and $\alpha_s\beta_s$, but a band with an intermediate mobility. No intermediate bands were observed in mixtures of $\alpha\beta$ and $\alpha_s\beta_s$ that had not been dissociated and reconstituted together. The intermediate band presumably corresponds to hybrid species, containing one native and one succinylated luciferase chain, in agreement with a dimeric structure for luciferase (Hastings *et al.*, 1969). Since the subunits in bacterial luciferase are nonidentical, such an intermediate band should in fact contain two types of hybrid molecules, one with a succinylated β chain and a native α chain ($\alpha\beta_s$) and the other with a succinylated α chain and a native β chain ($\alpha_s\beta$). The failure to resolve these two hybrid species is expected, since there should be no large differences in their electrophoretic mobilities. The relative amounts of $\alpha_s\beta$ and $\alpha\beta_s$ will depend on the concentrations of α_s and β_s, respectively, capable of being reconstituted to the dimeric structure. Only if these concentrations are identical in the succinylated luciferase sample ($\alpha_s\beta_s$) will the amounts of $\alpha\beta_s$ and $\alpha_s\beta$ be the same.

DEAE-Sephadex chromatography was conducted to further characterize the hybrid set, especially with regard to catalytic activity. Plots of optical density (A_{280}) and enzyme activity for chromatography of the hybrid set of native and succinylated luciferase are given in Figure 3. The optical density shows two small peaks at 46 and 80 ml, and two large unresolved peaks at about 110 ml. The fractions labeled I, II, and III were pooled, concentrated, and analyzed by cellogel electrophoresis (Figure 4), allowing them to be identified as native ($\alpha\beta$), hybrids ($\alpha\beta_s$ and $\alpha_s\beta$), and succinylated ($\alpha_s\beta_s$) luciferase, respectively. Relatively little material remains at the origin, indicating that the material observed at the origin in the previous electropherogram (Figure 2) was separated from the members of the hybrid set by DEAE-Sephadex chromatography. The minor peak at 46 ml was not identified.

The plot of luminescence shows activity associated both with reconstituted luciferase (I) and with the hybrid species (II), but succinylated luciferase (III) appears to be inactive. These results confirm the identity of peaks I and III as $\alpha\beta$ and $\alpha_s\beta_s$, respectively. The enzyme activity of the hybrids establishes that the combination of a native and a succinylated luciferase chain yields active or partially active molecules for at least one of the hybrids. The specific activity of the hybrid mixture ($\alpha\beta_s$ and $\alpha_s\beta$) is about 50% of that for reconstituted native luciferase. Whether the lower specific activity of hybrid mixtures is due to complete inactivity of one of the two hybrid species along with full activity of the other, or to partial activity of both can be determined by studying the species independently.

[3] The hybrid set refers to all the species and electrophoretic variants of luciferase with a dimeric structure, namely, $\alpha\beta$, $\alpha\beta_s$, $\alpha_s\beta$, and $\alpha_s\beta_s$.

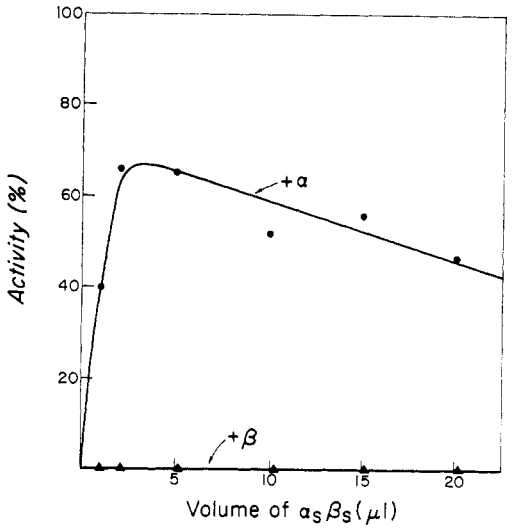

FIGURE 5: Complementation of the α (●) and β (▲) subunits of luciferase by succinylated luciferase ($\alpha_s\beta_s$). Succinylated luciferase was prepared by reaction of luciferase with 5.0 moles of succinic anhydride/lysyl residue. A constant amount of α (3.41×10^{-11} mole in 5 μl) or β (1.81×10^{-11} mole in 2 μl) subunits and varying amounts ($1-20 \times 10^{-10}$ mole in 1–20 μl) of $\alpha_s\beta_s$ were diluted from 5 M urea into 0.5 ml of 0.1 M phosphate–0.001 M EDTA–0.01 M DTT–0.2% BSA (pH 7.0) and reconstituted at 4° for 40 hr before measurement of luminescence activity. These activities were corrected for background luminescence introduced by $\alpha_s\beta_s$ (0.017 LU/μl). Control experiments in which the α subunit was titrated with excess β subunit and *vice versa* gave a maximum activity of 0.47 LU/l $\times 10^{-11}$ mole in both cases. The ordinate gives the percentage of maximum activity (4.7×10^{10} LU/mole or 600 LU/mg) and the abscissa the volume of $\alpha_s\beta_s$ added.

Complementation of Native α and β Subunits with $\alpha_s\beta_s$. Since the native luciferase subunits (α and β) can be separated by chromatography in 5 M urea (Gunsalus-Miguel *et al.*, 1972),[2] it is possible to utilize them individually with $\alpha_s\beta_s$ to reconstitute the different hybrid species, as described by

$$\alpha + \alpha_s + \beta_s \longrightarrow \alpha\beta_s + \alpha_s\beta_s$$

$$\beta + \alpha_s + \beta_s \longrightarrow \alpha_s\beta + \alpha_s\beta_s$$

At high molar ratios of $\alpha_s\beta_s$ to native subunit, the native subunit should recombine maximally with the complementing succinylated subunit to form the hybrid species ($\alpha\beta_s$ or $\alpha_s\beta$). Since the native subunits can be reconstituted to give luciferase with a high specific activity, it is possible to titrate a given amount of either α or β subunit with inactive succinylated luciferase (Figure 5). In these experiments, a fixed quantity of the native subunit and variable amounts of $\alpha_s\beta_s$ were mixed in 5 M urea and then reconstituted by dilution into buffer containing BSA. The ordinate gives the percentage of the maximum specific activity obtainable (600 LU/mg) if the same amount of α (or β) subunit was complemented with an excess of unmodified β (or α) subunit. The experimental points have been corrected for the background activity of $\alpha_s\beta_s$ (2%/μl).

The reconstitution of the α subunit with $\alpha_s\beta_s$ gives $\alpha\beta_s$ with a specific activity of up to 65% of that for reconstituted native luciferase. The negative slope for the plot of activity at high concentrations of $\alpha_s\beta_s$ reflects a dependence of reconstitutable activity on protein concentration. Such a dependence has also been observed for the native enzyme. Extrapolation of the slope to zero protein concentration gives a specific activity for $\alpha\beta_s$ of 70% of that for the reconstituted native enzyme. Reconstitution of β with $\alpha_s\beta_s$ (thus producing $\alpha_s\beta$) resulted in specific activities less than 1% of that for the native enzyme.

From these results we can conclude that the succinylation of luciferase results in the inactivation of the functional activity of the α subunit without greatly affecting the function of the β subunit. It may also be concluded that the subunits of luciferase have different functions.

Interpretation of these results may be complicated if the relative amounts of α_s and β_s capable of complementing with the native subunits are not equal in solutions of dissociated $\alpha_s\beta_s$. If more β_s than α_s is capable of being refolded, then the addition of even a large excess of $\alpha_s\beta_s$ to β will not result in complete complementation of the native subunit. In this case, the apparent specific activity of $\alpha_s\beta$ will be low. Similar considerations hold for the reconstitution to form $\alpha\beta_s$.

Purification of $\alpha\beta_s$ and $\alpha_s\beta$. Although the complementation experiments above gave the relative activities of $\alpha\beta_s$ and $\alpha_s\beta$, the specific activities of the hybrids could not be absolutely defined in these experiments; furthermore, further characterization of these species could not be conducted in the presence of a large excess of $\alpha_s\beta_s$. Accordingly, experiments were carried out to separate the hybrid species ($\alpha\beta_s$ or $\alpha_s\beta$) from $\alpha_s\beta_s$ and to remove unreconstituted subunits and aggregated material. The samples were prepared by reconstituting approximately equal molar amounts of native subunit and $\alpha_s\beta_s$. Although this will result in a large percentage of unreconstituted subunits, it is desirable to produce approximately equal amounts of the hybrid species and $\alpha_s\beta_s$, since it would be difficult to resolve the hybrid from large amounts of $\alpha_s\beta_s$.

Plots of optical density and enzyme activity *vs.* volume of eluent upon DEAE-Sephadex chromatography of reconstituted mixtures of α and $\alpha_s\beta_s$ and of β and $\alpha_s\beta_s$ are shown in Figure 6. The identity of the material eluted in the initial 80 ml is unknown, but in any event both the hybrids and the $\alpha_s\beta_s$ are well separated from contaminating material. No peak is present at the elution position (80 ml) of native luciferase (see Figure 3). However, in both chromatograms there are two overlapping peaks at the position of the hybrid species (about 95 ml) and $\alpha_s\beta_s$ (about 115 ml). The protein eluted at 95 ml in the upper chromatogram is the single hybrid species $\alpha\beta_s$ whereas in the lower column it is $\alpha_s\beta$.

The plot of luminescence shows a high peak of activity for $\alpha\beta_s$ (upper) whereas very little activity is found for $\alpha_s\beta$ (lower) or $\alpha_s\beta_s$ (both chromatograms). The maximum specific activity of the hybrid $\alpha\beta_s$ in this experiment was 500 LU/mg; that of $\alpha_s\beta$ was 40 LU/mg, and that of $\alpha_s\beta_s$ was less than 10 LU/mg. An extinction coefficient of 0.94 (0.1%, 1 cm) at 280 nm was used in these calculations for all modified species. These specific activities refer to the light units (LU) per milligram of modified luciferase with the dimeric structure of the native enzyme. Some of the values for the specific activities may be slightly low due to the fact that the modified species are not completely resolved.

The specific activity of 500 LU/mg for the $\alpha\beta_s$ hybrid in this experiment is in excellent agreement with the value obtained from the complementation titration experiments. However, an activity of 40 LU/mg for $\alpha_s\beta$ is somewhat higher than the value obtained previously. This latter result indicates that the percentage of β_s in $\alpha_s\beta_s$ capable of being reconstituted is greater than the percentage of α_s. The larger amount of $\alpha\beta_s$ reconstituted from α and $\alpha_s\beta_s$ (upper chromatogram) compared to the amount of $\alpha_s\beta$ reconstituted from β and $\alpha_s\beta_s$ (lower chromatogram) also supports this conclusion. Com-

parable amounts of the hybrids would be expected if the percentages of α_s and β_s capable of being reconstituted were identical, since the same number of moles of native subunit and $\alpha_s\beta_s$ were mixed in both samples. Although the above results show that the percentage of α_s capable of being reconstituted is less than β_s, at least 30% of the 1.5×10^{-7} mole of α_s in the mixture of β and $\alpha_s\beta_s$ was reconstituted, as measured by the amount of protein in the $\alpha_s\beta$ and $\alpha_s\beta_s$ bands (lower chromatogram). These results clearly establish that a luciferase molecule containing a succinylated α chain and a native β chain and maintaining the quaternary structure of the native enzyme is virtually inactive. Since $\alpha\beta_s$ has a relatively high specific activity, the subunits of luciferase must have different functions in the bioluminescent reaction.

Discussion

From recent studies it is becoming increasingly evident that not only do a large number of proteins exist as oligomers containing two or more subunits; in many cases the subunits are nonidentical (Klotz *et al.*, 1970; Klotz and Darnall, 1969; Ginsberg and Stadtman, 1970). Aspartate transcarbamylase, hemoglobin, and RNA polymerase are examples of such heteropolymeric proteins. However, even more complicated structures, such as the multienzyme complexes (Lynen, 1969; Reed, 1969) and the ribosomes (Kurland *et al.*, 1969; Nomura *et al.*, 1969), and possibly even membranes and cell organelles, might be similarly viewed. Actually many proteins that have been isolated and studied as individual species *in vitro* may exist and function *in vivo* in association with other proteins or macromolecular components (Kempner and Miller, 1968a,b). Consequently the function and regulation of many catalytic, structural, and transport proteins *in vivo* may be expected to be greatly influenced by heterologous subunit interactions.

Bacterial luciferase is an unusually advantageous protein for studying such phenomena. Its dimeric structure, with two distinctly different subunits, is representative of the simplest type of heteropolymeric protein, its behavior being uncomplicated by the presence of possible homologous subunit interactions found in more complicated heteropolymeric proteins. In addition, the activity of luciferase can be measured over a very wide range, between 10^{-2} and 10^{-10} g (Mitchell and Hastings, 1971). Finally, kinetics and spectral properties of the light reaction may provide a sensitive probe for conformational changes.

Hybridization of native and chemically modified proteins can be used to study subunit structure and interactions. Suitable chemically modified variants having the same quaternary structure as the native enzyme yet differing in charge have been previously prepared for aldolase (Meighen and Schachman, 1970a), GPDH (Meighen and Schachman, 1970b), and ATCase (Meighen *et al.*, 1970a) by limited reaction with succinic anhydride. The preparation is simple and rapid; succinylation of the protein requires less than 20 min under mild conditions. Approximately 25–50% of the lysyl residues are modified. The homogeneity, charge and relative molecular weight of different succinylated preparations can be readily examined after cellulose acetate electrophoresis for about 20 min. Thus, the production of succinylated derivatives and the analysis for a variant suitable for hybridization experiments is a simple and rapid process that should be generally applicable to a wide variety of oligomeric proteins.

Quaternary structure and function have also been studied with genetic variants (Lew and Roth, 1971). The method does have limitations, however, and there are at the same time im-

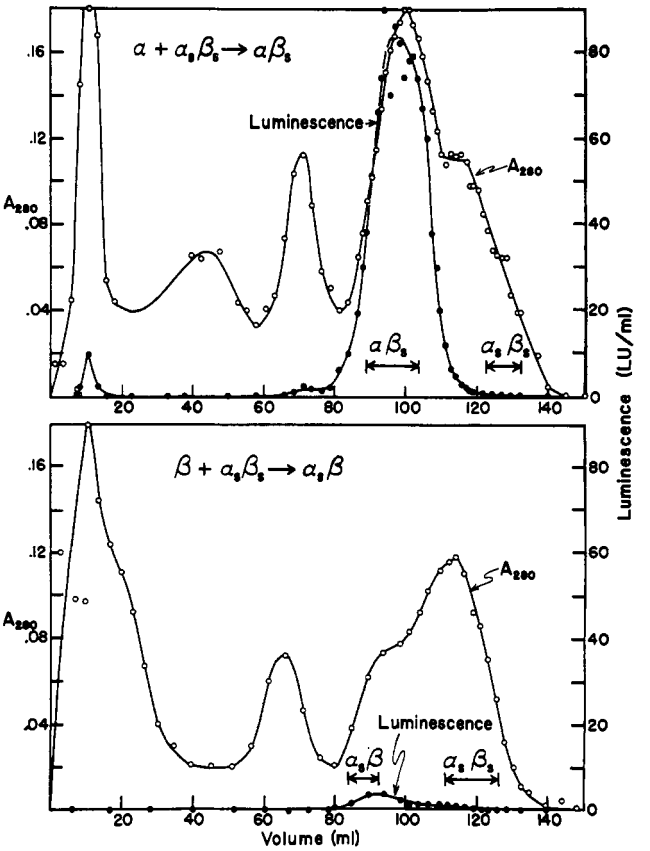

FIGURE 6: Purification of $\alpha\beta_s$ and $\alpha_s\beta$ by DEAE-Sephadex chromatography. Succinylated luciferase (1.5×10^{-7} mole) was mixed in 3–4 ml of 5 M urea–0.02 M DTT–0.001 M EDTA–0.04 M phosphate buffer (pH 7.0), 4°, containing 1.1×10^{-7} mole of either α or β subunit. The resulting mixtures were diluted into 50 ml of 0.001 M EDTA—0.1 M phosphate buffer (pH 7.0) and dialyzed exhaustively *vs.* the same buffer containing 2×10^{-4} M DTT at 4°. The dialysis bags were embedded in solid Aquacide II (Calbiochem) at 4° until the sample volume decreased to 4–5 ml (about 12–24 hr) and then were equilibrated at 4° in 0.2 M NaCl–0.001 M EDTA–0.05 M Tris-chloride (pH 7.5). Chromatography on DEAE-Sephadex was conducted as described in Figure 3, except the buffers contained 2×10^{-4} M DTT. The fractions $\alpha\beta_s$, $\alpha_s\beta$, and $\alpha_s\beta_s$ were pooled as indicated. A_{280}: ○; LU/ml: ●.

portant differences and some advantages in using chemically modified enzymes for such studies. First, the hybridization of genetic variants is limited to (a) enzymes for which two naturally occurring electrophoretic variants are readily available (Shaw, 1964; Markert and Whitt, 1968) and (b) bacterial enzymes that can be readily mutated and screened for the desired electrophoretic properties (Lew and Roth, 1971). This latter condition generally requires the use of a specific stain for that enzyme. In contrast, chemical variants of a pure enzyme can be rapidly prepared, and protein stains can be used to detect the members of the hybrid set upon electrophoresis. Secondly, many experiments with genetic variants have involved "*in vivo*" hybridization procedures, but the interpretation of those results may be complicated because variants may arise from causes other than the combination of different subunits in oligomeric proteins (Markert and Whitt, 1968). With *in vitro* hybridization, the production of various molecular forms can be directly related to the exchange of subunits. Conditions favorable for the dissociation and reconstitution for many oligomeric proteins are well known (Sund and Weber, 1966; Anfinsen, 1968; Teipel and Koshland, 1971). Fi-

nally, genetic variants are usually selected as being functionally active, whereas in most cases the succinylated variants are enzymatically inactive. In addition, some genetic variants cannot be hybridized; apparently the subunit interactions are sufficiently different that no active hybrid species can form (Kaplan, 1964).

A major goal of the present investigations on luciferase was the elucidation of the functional significance of the nonidentical polypeptide chains of luciferase, both of which are known to be required for catalytic activity in the bioluminescent reaction (Friedland and Hastings, 1967). A considerable number of proteins have been found to contain more than one type of polypeptide chain (Ginsberg and Stadtman, 1970). These may have similar functions, as in hemoglobin, or they may be involved in different catalytic steps, as in tryptophan synthetase (Crawford and Yanofsky, 1958) and also the larger multienzyme complexes (Reed, 1969; Lynen, 1969). Alternatively, the protein may consist of both catalytic and noncatalytic subunits as in ATCase, where one type is catalytic and the other is involved in regulation (Gerhart and Schachman, 1965), or in lactose synthetase, where the second subunit (α-lactalbumin) modifies the specificity of the catalytic chains (Brew *et al.*, 1968). RNA polymerase from *Escherichia coli* may be another protein of this type (Burgess *et al.*, 1969).

The present experimental results clearly show that the subunits of luciferase have different functions, since $\alpha_s\beta_s$ and $\alpha_s\beta$ were essentially inactive whereas $\alpha\beta_s$ and $\alpha\beta$ both had high activity. Further work will be required to deduce whether each subunit is catalytically functional or whether one subunit is catalytic while the other has a nonenzymatic role. A subsequent paper investigates the functional properties of the above hybrid set in an attempt to deduce more clearly the functional importance of the two subunits in bacterial luciferase.

References

Anfinsen, C. B. (1968), *Develop. Biol., Suppl. 2*, 1.

Brew, K., Vanaman, T. C., and Hill, R. L. (1968), *Proc. Nat. Acad. Sci. U. S. 59*, 491.

Burgess, R. R., Travers, A. A., Dunn, J. J., and Bautz, E. K. F. (1969), *Nature (London) 221*, 43.

Cohn, E. J., and Edsall, J. T. (1943), Proteins, Amino Acids and Peptides, New York, N. Y., Reinhold, p 370.

Crawford, I. P., and Yanofsky, C. Y. (1958), *Proc. Nat. Acad. Sci. U. S. 44*, 1161.

Fraenkel-Conrat, G. (1957), *Methods Enzymol. 4*, 247.

Friedland, J. M., and Hastings, J. W. (1967), *Proc. Nat. Acad. Sci. U. S. 58*, 2336.

Gerhart, J. C., and Schachman, H. K. (1965), *Biochemistry 4*, 1054.

Ginsberg, A., and Stadtman, E. R. (1970), *Annu. Rev. Biochem. 39*, 429.

Hastings, J. W. (1968), *Annu. Rev. Biochem. 37*, 597.

Hastings, J. W., and Gibson, Q. H. (1963), *J. Biol. Chem. 238*, 2537.

Hastings, J. W., and Weber, G. (1963), *J. Opt. Soc. Amer. 53*, 1410.

Hastings, J. W., Weber, K., Friedland, J., Eberhard, A., Mitchell, G. W., and Gunsalus, A. (1969), *Biochemistry 8*, 4681.

Kaplan, N. O. (1964), *Brookhaven Symp. Biol. 17*, 131.

Kempner, E. S., and Miller, J. M. (1968a), *Exp. Cell Res. 51*, 141.

Kempner, E. S., and Miller, J. M. (1968b), *Exp. Cell Res. 51*, 150.

Klotz, I. M., and Darnall, D. W. (1969), *Science 166*, 126.

Klotz, I. M., Langerman, N. R., and Darnall, D. W. (1970), *Annu. Rev. Biochem. 39*, 25.

Kurland, C. G., Voynow, P., Hardy, S. J. S., Randall, L., and Lutter, L. (1969), *Cold Spring Harbor Symp. Quant. Biol. 34*, 17.

Lew, K. K., and Roth, J. R. (1971), *Biochemistry 10*, 204.

Lynen, F. (1969), *Perspectives Biol. Med. 12*, 204.

Markert, C. L., and Whitt, G. S. (1968), *Experientia 24*, 977.

Meighen, E. A., Pigiet, V., and Schachman, H. K. (1970a), *Proc. Nat. Acad. Sci. U. S. 65*, 234.

Meighen, E. A., and Schachman, H. K. (1970a), *Biochemistry 9*, 1163.

Meighen, E. A., and Schachman, H. K. (1970b), *Biochemistry 9*, 1177.

Meighen, E. A., Smillie, L. B., and Hastings, J. W. (1970b), *Biochemistry 9*, 4949.

Mitchell, G. W., and Hastings, J. W. (1971), *Anal. Biochem. 39*, 243.

Moore, S., and Stein, W. G. (1948), *J. Biol. Chem. 176*, 367.

Nomura, M., Mizushima, S., Ozaki, M., Traub, P., and Lowry, C. V. (1969), *Cold Spring Harbor Symp. Quant. Biol. 34*, 49.

Reed, L. J. (1969), *Curr. Top. Cell. Reg. 1*, 233.

Shaw, C. R. (1964), *Brookhaven Symp. Biol. 17*, 117.

Sund, H., and Weber, K. (1966), *Angew. Chem., Int. Ed. Engl. 5*, 231.

Teipel, J. W., and Koshland, D. E. (1971), *Biochemistry 10*, 792.

Ziegler, M., Meighen, E. A., and Hastings, J. W. (1971), *Fed. Proc., Fed. Amer. Soc. Exp. Biol. 30*, 1057 Abs.