Functional Differences of the Nonidentical Subunits of Bacterial Luciferase. Properties of Hybrids of Native and Chemically Modified Bacterial Luciferase*

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ABSTRACT: Experiments were carried out to investigate the specific role of each of the nonidentical subunits of bacterial luciferase by comparing the properties of the members of the hybrid set of native ($\alpha\beta$) and succinylated ($\alpha_s\beta_s$) luciferase. Properties examined were the pH–activity profile, thermal stability, lifetime of an enzyme–substrate intermediate (turnover rate), and the binding of reduced flavin mononucleotide. It was found that whereas physical properties of the protein exhibit alterations if either of the two subunits is modified, properties which are more specifically associated with the reactive center are altered only in molecules possessing an altered α subunit. The α subunit thus appears to participate directly in the catalytic steps of the bioluminescent reaction. The β subunit, however, seems not to be so intimately involved in the actual reaction steps; though this indicates it may have some other, possibly noncatalytic role, its exact function remains unknown.

Bacterial luciferase has been shown to be a dimeric heteropolymeric protein, designated $\alpha\beta$, in which the two subunits are functionally different (Hastings, 1968; Meighen *et al.*, 1971). The functional difference has been deduced from studies of luciferase which has been chemically modified by limited reaction with succinic anhydride (designated $\alpha_s\beta_s$). While this species has been found to be inactive, hybrid molecules, possessing one native and one succinylated subunit, differ markedly in activity: the species with a modified α subunit ($\alpha_s\beta$) is virtually inactive (as is the fully succinylated luciferase) while $\alpha\beta_s$ has considerable activity, at least half that of the native enzyme.

In the experiments reported here, properties of each of the members of the hybrid set have been compared in some detail. It has been found that whereas physical properties of the protein (notably thermal stability) exhibit alterations if either of the two subunits is modified, properties which are more specifically associated with the reactive center (especially the lifetime of the enzyme–substrate intermediate) are altered only in molecules possessing an altered α subunit. The importance of the α subunit in the bioluminescent reaction is thus established; the function of β remains unknown.

Experimental Section

Native luciferase was isolated from the marine luminous bacterium, *Photobacterium fischeri*, strain MAV, grown in a complex medium (Hastings *et al.*, 1969). Pure luciferase and the individual subunits were isolated as previously described (Gunsalus-Miguel *et al.*, 1972).[1] Succinylated luciferase ($\alpha_s\beta_s$) was prepared by reaction of native luciferase ($\alpha\beta$) with 4.5 moles of succinic anhydride/lysyl residue (Meighen *et al.*, 1971). The hybrid species ($\alpha\beta_s$ or $\alpha_s\beta$) were obtained by mixing unmodified α or β subunits (separated by DEAE-Sephadex chromatography in 5 M urea) with $\alpha_s\beta_s$ in 5 M urea and allowing the hybrid species to reconstitute by removing the urea. Purification was achieved by DEAE-Sephadex chromatography. Purified reconstituted $\alpha_s\beta_s$ was also obtained by this procedure. Both native and succinylated luciferases were dissociated in urea and reconstituted to provide parental species that had been reconstituted from subunits. Additional details are given in the preceding paper (Meighen *et al.*, 1971).

The members of the hybrid set are designated as $\alpha\beta$, $\alpha\beta_s$, $\alpha_s\beta$, and $\alpha_s\beta_s$; the specific activities of each are given in Table I. The experiments described below were conducted within a few weeks after isolation and the same preparations used in all experiments. During this period activity losses were less than 30% for any member of the hybrid set. Although substantial loss of activity occurred after storage for 2 months at 4°, no consequent effect was observed on the properties below which were reinvestigated at that time.

The reaction catalyzed by luciferase involves the bioluminescent oxidation of $FMNH_2$[2] by molecular oxygen. Reaction rate is measured instantaneously and continuously during the course of the reaction by the light intensity, in quanta per second. The rate and yield of photon emission is greatly affected by long-chain aliphatic aldehydes (Hastings *et al.*, 1963, 1969).

The standard *in vitro* assay for luciferase activity was carried out by rapidly injecting from a syringe, 1 ml of catalytically reduced FMN (5×10^{-5} M) into a reaction mixture (1.2 ml) containing luciferase, aldehyde (15 μl of a 0.1% (v/v) sonicated suspension of dodecanal unless otherwise noted), and oxygen in 0.2% BSA–0.02 M phosphate buffer at pH 7. Light intensity rises rapidly (<1 sec) to its maximum and decays exponentially with a characteristic lifetime of several seconds;

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[2] Abbreviations used are: $FMNH_2$ and FMN, reduced and oxidized riboflavin 5′-phosphate; BSA, bovine serum albumin; ATCase, aspartate transcarbamylase.

TABLE I: Activity of the Hybrid Set of Native and Succinylated Luciferase.

Luciferase Variant	Sp Act. (LU/mg)
$\alpha\beta$	
Native	1000
Dissociated and reconstituted	600–800
$\alpha\beta_s$	400–500
$\alpha_s\beta$	20–40
$\alpha_s\beta_s$	
Untreated	10[a]
Dissociated and reconstituted and purified	5–10

[a] Based on the molar concentration of molecules with a dimeric structure.

the exact half-time is dependent upon several factors, including temperature and chain length of the aldehyde. As will be discussed in greater detail in a later section, the free substrate itself has a lifetime of less than a second, so that the observed lifetime of light emission must be attributed to an enzyme–substrate intermediate. Such a long lifetime is not only very unusual for an enzyme–substrate intermediate; it provides an unusually sensitive probe and index of active site chemistry.

Light intensity was measured with a photometer similar to that described by Mitchell and Hastings (1971) utilizing a photomultiplier tube (1-P21). The calibration was made in quanta sec^{-1}, using the standard of Hastings and Weber (1963).

Results

Effect of pH on the Activity. The effects of pH on the relative activities of the members of the hybrid set of native and succinylated luciferase ($\alpha\beta$, $\alpha\beta_s$, $\alpha_s\beta$, and $\alpha_s\beta_s$) are given in Figures 1 and 2. The experimental activities have been normalized to the point of optimal activity for each variant. In the lower pH regions, the activity profiles of $\alpha\beta_s$ and $\alpha_s\beta$ are shifted to higher pH values relative to $\alpha\beta$ in both the Tris-citrate (Figure 1) and phosphate (Figure 2) buffers. The activity profile of $\alpha_s\beta_s$ in this region is shifted to a greater extent than the profiles of either hybrid species containing only one modified subunit. The relative effects are similar but not identical in the two buffer systems presumably reflecting differences in the buffer composition.

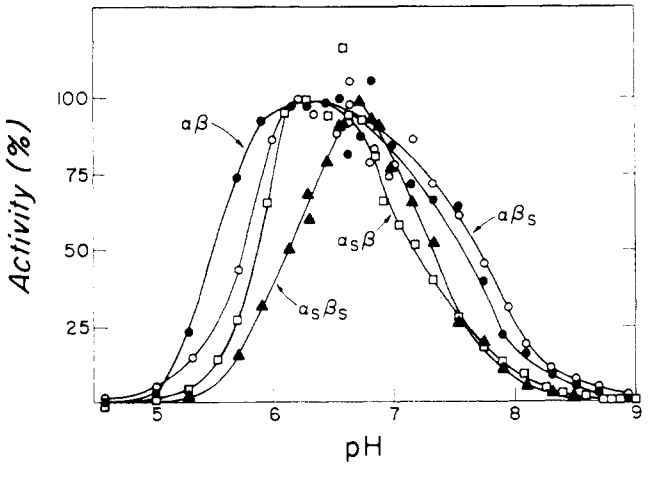

FIGURE 1: Effect of pH on the activities of the members of the hybrid set in Tris-citrate buffer. $\alpha\beta$(●), $\alpha\beta_s$ (○), $\alpha_s\beta$ (□), and $\alpha_s\beta_s$ (▲). Stock 1 M solutions of Tris base and citric acid were mixed in different proportions to achieve the particular pH value and then diluted 1:50 into 1.2 ml of the standard assay mix. The pH of each solution was measured immediately after injection of the $FMNH_2$ in the standard assay. The ordinate gives the initial maximum light intensity as per cent of the highest value for each variant. Temperature 23°.

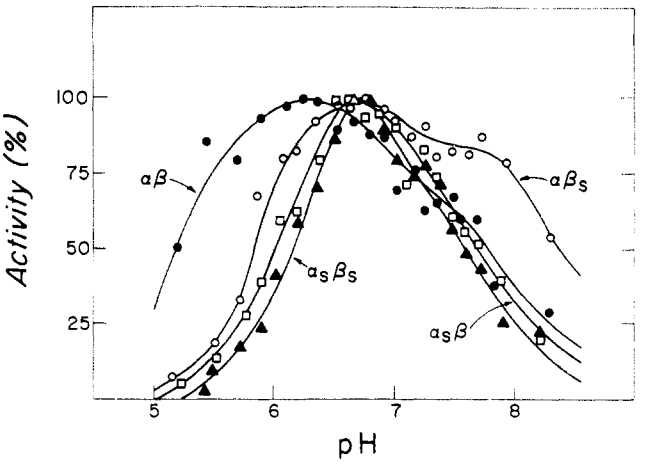

FIGURE 2: Effect of pH on the activities of the members of the hybrid set in phosphate buffers, designated as in Figure 1. Stock 1 M solutions of NaH_2PO_4 and K_2HPO_4 were mixed in different proportions to achieve the particular pH value and were then diluted 1:50 into 1.2 ml of the standard assay mix. Other details are given in the legend to Figure 1.

In the upper pH regions, only the activity profile of $\alpha\beta_s$ has been shifted to higher pH values relative to $\alpha\beta$. The effect is especially dramatic in phosphate buffer. This effect, as well as the shifts for the variants in the lower pH region, might be due to electrostatic interaction of the negatively charged succinyl groups with other polar residues; on the other hand, the effects might simply arise from a decrease in the pH in the microenvironment of the protein resulting from the introduction of the succinyl groups. That is, the negatively charged succinyl groups would tend to repel OH^- and attract H_3^+O making the pH of the microenvironment of the variants effectively lower than that of the native enzyme.

The shifts of the activity profiles of $\alpha_s\beta$ and $\alpha_s\beta_s$ in the upper pH regions cannot simply be explained on the basis of a change in the pH near the protein. The profiles of both $\alpha_s\beta$ and $\alpha_s\beta_s$ are shifted to lower pH values relative to $\alpha\beta$. These shifts are substantially larger in the Tris-citrate than in the phosphate buffer. The presence of succinyl groups on the α subunits, in particular, has apparently destabilized luciferase in the upper pH region whereas succinyl groups on the β subunit have little effect on this regard. For example, compare the relative activities of $\alpha_s\beta_s$ and $\alpha_s\beta$ to $\alpha\beta_s$ and $\alpha\beta$ in the upper pH regions. If we further assume that the pH in the microenvironment of the variants is lower than that for $\alpha\beta$, the destabilization by the succinyl groups on the α subunit is actually even greater. The apparent destabilization of luciferase at high pH can perhaps be considered as a direct effect whereas the pH changes in the microenvironment of the protein are indirect effects arising from the presence of the negatively charged succinyl groups. Although the above explanation is certainly oversimplified, its predictions adequately describe the changes in

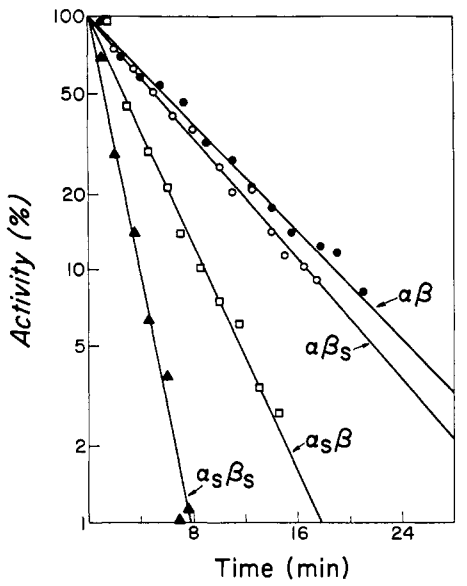

FIGURE 3: Rates of thermal inactivation at 45° of each of the members of the hybrid set of native and succinylated luciferase, prepared as described in the text. The members of the hybrid set (designated as in Figure 1) were incubated at 45° at a concentration of about 0.02 mg/ml, in 0.1 M phosphate buffer (pH 7.0) with 0.001 M EDTA and 0.1% BSA. Aliquots were removed at the times indicated, rapidly cooled, and assayed for bioluminescent activity. Ordinate, percentage of the initial activity, on a logarithmic scale; abscissa, time of incubation.

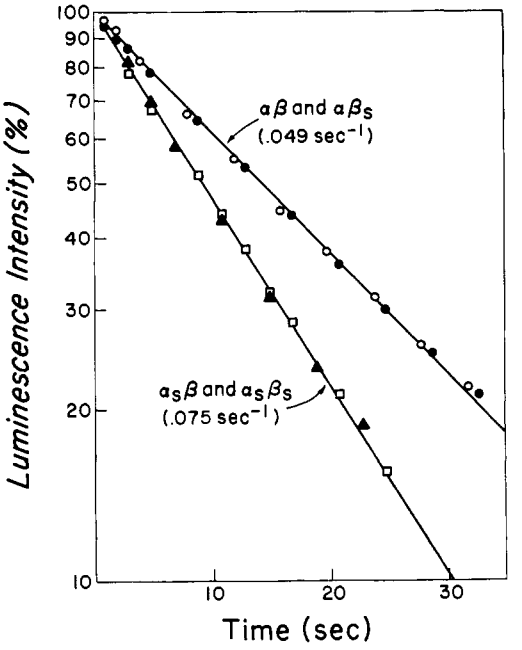

FIGURE 4: Kinetics of the decay of luminescence for the members of the hybrid set at 25° [$\alpha\beta$ (●), $\alpha\beta_s$ (○), $\alpha_s\beta$ (□), and $\alpha_s\beta_s$ (▲)]. In the assay (see Experimental Section) octanal (5 μl of a 0.1% (v/v) sonicated suspension) was used in place of dodecanal. Ordinate, per cent of maximum light intensity on a logarithmic scale; abscissa, time after injection of $FMNH_2$. The values of the first-order rate constant for the decay of luminescence are given for each plot in sec^{-1}.

the pH–activity profiles arising from the introduction of the succinyl groups on either subunit of luciferase.

Temperature Sensitivity of the Hybrid Set. The loss of luciferase activity upon incubation at 45° is shown in Figure 3 for each of the members of the hybrid set. The fact that inactivation is exponential with time indicates that each of the modified species is relatively homogeneous. The first-order rate constants of inactivation for $\alpha\beta$, $\alpha\beta_s$, $\alpha_s\beta$, and $\alpha_s\beta_s$ are 0.12, 0.13, 0.26, and 0.57 min^{-1}, respectively. The introduction of succinyl groups, particularly onto the α subunit, has made the luciferase molecule more sensitive to temperature inactivation. The inactivation rate of $\alpha_s\beta_s$ is somewhat greater than might be predicted from the effects of the individual succinylated chains in $\alpha\beta_s$ and $\alpha_s\beta$.

The effect of dissociation and reconstitution on the temperature sensitivity of $\alpha\beta$ and $\alpha_s\beta_s$ was also investigated. The inactivation rate of $\alpha_s\beta_s$ that had undergone the same treatment as the hybrid species (dissociation, reconstitution, and chromatography) was similar (within experimental error) to that of the untreated $\alpha_s\beta_s$ (k = 0.48 and 0.57 min^{-1}, respectively). Dissociation and reconstitution of native luciferase did not affect its temperature sensitivity (k = 0.12 min^{-1}). Thus, the variation in temperature sensitivity among the members of the hybrid set must arise entirely from the introduction of the negatively charged succinyl groups.

Decay Kinetics of Light Emission. Bioluminescence is measured *in vitro* by the rapid injection of $FMNH_2$ into an assay solution containing the enzyme and a long-chain aliphatic aldehyde. The light intensity rises rapidly to a maximum and then decays exponentially. Since the excess substrate is oxidized nonenzymatically within the first second, while light persists for a considerably longer period, a stable intermediate must be formed. This intermediate decays at a first-order rate, the specific value of which is greatly affected by the chain length of aldehyde (Hastings *et al.*, 1963, 1969).

The time course for this decay of luminescence, using octanal, is given in Figure 4 for each of the members of the hybrid set. The light intensity is plotted on a log scale as a function of time and gives a linear relationship for each luciferase variant, again indicating the homogeneity of each of the species. The first-order rate constants for $\alpha\beta$ and $\alpha\beta_s$ are identical (0.049 sec^{-1}), whereas the rate constants for both $\alpha_s\beta$ and $\alpha_s\beta_s$ are 0.075 sec^{-1}. The differences between these values and those given in Table II (see below) arise from the use of nonsaturating amounts of octanal in the experiment shown in Figure 4. Under these conditions, all decay rates were greater than

TABLE II: Rate Constants for the Decay of Luminescence for Members of the Hybrid Set with Different Aldehydes.[a]

Luciferase Variant	Octanal	Decanal	Dodecanal
$\alpha\beta$	42.3 ± 3	267 ± 17	42.7 ± 3
$\alpha\beta_s$	41.8 ± 3	277 ± 17	41.3 ± 4
$\alpha_s\beta$	57.5 ± 5	300 ± 17	50.0 ± 4
$\alpha_s\beta_s$	57.5 ± 5	300 ± 17	52.7 ± 4

[a] The decay of light intensity was measured in the standard assay system at saturating aldehyde concentrations. The numerical values give the average first-order rate constants in $sec^{-1} \times 10^3$ for the decay of light intensity and the maximum deviations of at least five independent determinations. All measurements for a particular aldehyde were conducted within a few hours and the assays for the members of the hybrid set interspersed. This approach reduces deviations arising from the slight differences in temperature (22–24°).

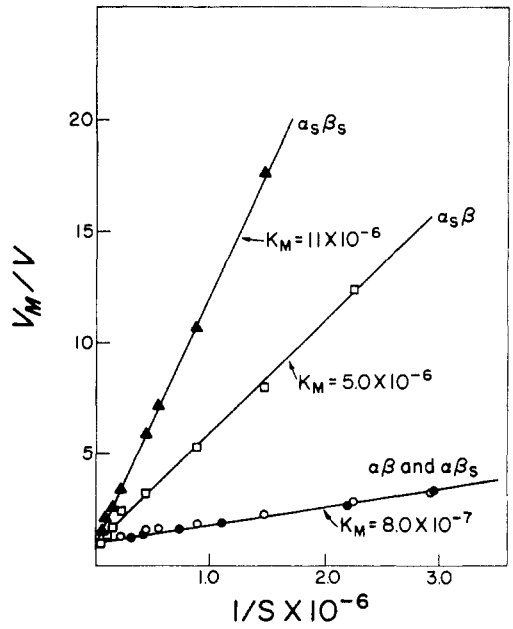

FIGURE 5: The effect of $FMNH_2$ concentration on initial light intensity for the members of the hybrid set. The initial maximum light intensity at saturating substrate ($FMNH_2$) concentration (V_m) divided by the initial light intensity at the particular substrate concentrations (v) is plotted *vs.* the reciprocal of the $FMNH_2$ concentration. The assay was initiated at 23° by injection of 1.0 ml of 0.005% (v/v) sonicated suspension of decanal into 2.2 ml of 0.01 M phosphate containing 0.2 ml of 1% BSA, FMN at the indicated concentrations, and enzyme. About 0.5 mg of $Na_2S_2O_4$, which reduces the flavin in the vial, was added to the assay mix as a solid just prior to injection of the aldehyde suspension. The reaction is initiated by virtue of the excess oxygen dissolved in the aldehyde suspension. The amount of $Na_2S_2O_4$ added could be varied over a reasonable range (about 0.3–0.8 mg) without affecting the initial light intensity.

those with saturating aldehyde. The fact that succinyl groups have no effect on the decay rate when present on the β subunit, but are effective on the α subunit, indicates that the long-lived enzyme–substrate intermediate is specifically associated with the α subunit only. The increase in decay rate in $\alpha_s\beta$ and $\alpha_s\beta_s$ relative to $\alpha\beta$ can be viewed both as an increase in the turnover rate and a destabilization of the enzyme intermediate by the succinyl groups on the α subunit.

With other aldehydes similar, but quantitatively different results have been obtained. The effect of three different aldehydes on the decay of the light intensity of the members of the hybrid set is given in Table II. For any given aldehyde, the decay rates for $\alpha\beta$ and $\alpha\beta_s$ are identical as are the rates for $\alpha_s\beta$ and $\alpha_s\beta_s$. The first-order rate constants for $\alpha_s\beta$ and $\alpha_s\beta_s$ are 40, 10, and 20% greater than those of $\alpha\beta$ and $\alpha\beta_s$ for octanal, decanal, and dodecanal, respectively. Since each rate constant has an average deviation of ±5%, the variation in the rate constants for decanal are not much greater than experimental error. However, the differences in decay rates with octanal and dodecanal are certainly significant.

It might be assumed that the longer the chain length of the aliphatic aldehyde, the greater the possibility of succinyl groups on luciferase affecting the decay rate relative to that for $\alpha\beta$. However, no apparent trend can be observed in the present experiments as the chain length of the aldehyde is increased. In fact, the succinyl groups have very little effect on the rela-decay rates with decanal whereas the rates have been increased significantly both with octanal and dodecanal in variants containing the succinylated α subunit. This result might suggest that the exact conformation of the stable light-emitting intermediate is substantially affected by the chain length of the aldehyde.

Binding of $FMNH_2$. Recent experimental investigations on native luciferase have shown that the bioluminescent reaction shows a first-order dependence on the $FMNH_2$ concentration, with a K_m of 8×10^{-7} M (Meighen and Hastings, 1971). These results were obtained using a different method for initiation of the bioluminescent reaction. Instead of injecting the catalytically reduced $FMNH_2$ into the luciferase solution as in the standard assay, the enzyme and flavin are first mixed with a slight excess of $Na_2S_2O_4$ in order to remove free oxygen and reduce the flavin. The bioluminescent reaction is then initiated by the injection of 1 ml of an aqueous solution of the long-chain aliphatic aldehyde, with dissolved O_2 present. The light intensity obtained by this method at saturating $FMNH_2$ concentrations was identical with that obtained in the standard assay, and at the same time gave very reproducible light intensities over a wide range of $FMNH_2$ concentrations, down to 5×10^{-9} M. Using this technique we were able to carry out a comparative study of the binding of $FMNH_2$ with the members of the hybrid set.

A plot of V_m/v *vs.* $1/FMNH_2$ concentration is given in Figure 5 for the different luciferase variants. Linear plots are obtained for each species, thus again demonstrating the relative homogeneity of the preparations. The K_m values for $\alpha\beta$ and $\alpha\beta_s$ are identical, 8.0×10^{-7} M. However, those for $\alpha_s\beta$ and $\alpha_s\beta_s$ are substantially different, with values of 5.0×10^{-6} and 11×10^{-6} M, respectively.

The experimental results thus show that the substitution in luciferase of α_s for a native α subunit decreases the binding of $FMNH_2$ 5- to 10-fold. However, the replacement of the β subunit by β_s has little effect on the binding of $FMNH_2$. This result suggests that the binding site for $FMNH_2$ may be on the α subunit.

Discussion

The major conclusion to be drawn from the experiments reported is that the chemical steps in the luminescent reaction are quite probably occurring specifically on the α subunit.

Since the decay time of luminescence relates to the lifetime (stability) of the enzyme–substrate intermediate, the half-time for luminescence is presumed to be highly diagnostic of and sensitive to effects related specifically to the active site and its chemistry. The fact that the presence of an altered α subunit results in an altered lifetime of luminescence whereas β has no effect suggests that the α subunit is specifically involved in the reaction. A similar specificity occurs with respect to the K_m for flavin binding: the hybrid with the altered β is indistinguishable from the native enzyme, while the hybrid with an altered α exhibits a K_m higher by a factor of greater than six. There is an apparent (but small) effect of the defective β on the K_m of the fully succinylated luciferase.

With regard to effects of modified subunits upon the other parameters measured, less specificity is to be expected for probes such as thermal stability or the pH-activity profile, which may rely more on the integrity of general protein structure. It is nevertheless true that in these cases also the presence of a modified β in the hybrid appears to have less drastic effects than does an altered α. The reason for this is not clear but it may be of significance in relation the function of the β subunit.

The above interpretation of the results is based on the as-

sumption that the chemical modification of one subunit would not greatly affect the behavior of the (unmodified) complementing subunit. The argument might be made that such an assumption is not justifiable; for example, if there were flavin-binding sites on the β subunit, they might be affected by succinylation of the α subunit. There are, of course, proteins in which the regulation of the activity of a catalytic subunit occurs normally *via* the interaction of an effector molecule and an associated regulatory subunit. But chemical modification, as with succinic anhydride, is presumably far less specific and unlikely to generate such forces between subunits. Experiments on aldolase (Meighen and Schachman, 1970a), glyceraldehyde-3-phosphate dehydrogenase (Meighen and Schachman, 1970b), and ATCase (Meighen *et al.*, 1970) have shown that the functional activity of one subunit is not substantially altered by the presence of succinyl groups on another subunit in the same oligomer. Moreover, an ATCase molecule containing one active catalytic subunit and one inactive succinylated catalytic subunit and native regulatory subunits retained both homotropic and heterotropic effects (Pigiet *et al.*, 1970). The above evidence thus supports the viewpoint that the limited succinylation of one subunit in luciferase will not substantially alter the specific function of the complementing subunit.

It seems highly probable from the present experiments that the flavin-binding and light-emitting steps occur on the α subunit rather than on the β subunit. Since these steps are presumably the initial and final steps in the *in vitro* bioluminescent reaction, it might be tentatively concluded that all the chemical steps in the reaction occur on the α subunit. There is presently no satisfactory hypothesis concerning the role of the β subunit. Although a catalytic role has not been completely eliminated, the present results suggest that the β subunit may play a noncatalytic but functional role in the reaction. Several heteropolymeric proteins have been shown to contain noncatalytic subunits, including aspartate transcarbamylase (Gerhart and Schachman, 1965), lactose synthetase (Brew *et al.*, 1968), and RNA polymerase from *E. coli* (Burgess *et al.*, 1969). The β subunit of luciferase may likewise have some regulatory or modifier function or it may simply serve to maintain the solubility and conformation of the α subunit. However, since no functional activity has yet been assigned to either the α or β subunit alone (Friedland and Hastings, 1967), the above conclusions may be premature without extensive investigations of other steps in the bioluminescent reaction with a variety of genetic and chemical variants of luciferase altered in one or both subunits.

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