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Characterization of the Aldehyde Binding Site of Bacterial Luciferase by Photoaffinity Labeling†

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ABSTRACT: A photoaffinity probe 1-diazo-2-oxoundecane has been synthesized and used to examine the aldehyde-binding site of the nonidentical dimeric luciferase ($\alpha\beta$) from *Vibrio harveyi* cells. In the dark, the probe competes against aldehyde in binding to luciferase. Irradiation of luciferase and the probe at 254 nm resulted in primarily specific labeling of both α and β subunits with concomitant enzyme inactivation, but significant ($\simeq$40%) nonspecific labeling of mainly the β subunit also occurred. The addition of decanal to protect the active center reduced the rate of inactivation. When 2-mercaptoethanol was included to quench the nonspecific labeling, the amounts of probe incorporated into α and β correlated stoichiometrically with the quantities of enzyme photoinactivated. On the basis of these findings, we postulate that the aldehyde binding site is at or near the subunit interface of luciferase.

Bacterial luciferase catalyzes the monooxygenation reaction in which reduced FMN ($FMNH_2$)[1] and a long-chain aliphatic aldehyde react with O_2 to yield FMN, carboxylic acid, water, and light (Hastings & Nealson, 1977; Ziegler & Baldwin, 1981, and references therein). The luciferases from at least four strains of marine bacteria, including *Vibrio harveyi* (Baumann et al., 1980) previously designated as *Beneckea harveyi* (Reichelt & Baumann, 1973), have been purified, and each was shown to be a nonidentical dimer with the heavy and light subunits referred to as α and β, respectively (Hastings et al., 1969; Ruby & Hastings, 1980; Meighen & Bartlet, 1980). Earlier hybridization studies using chemically (Meighen et al., 1971) and mutationally (Cline & Hastings, 1972) modified *V. harveyi* luciferase subunits indicated that α (M_r 42 000) participated directly in catalysis whereas the specific function of β (M_r 37 000) remained unclear. Selective modifications of the α subunit by sulfhydryl (Nicoli et al., 1974) and histidyl (Cousineau & Meighen, 1976) reagents or proteases (Baldwin et al., 1978; Holzman et al., 1980) were subsequently shown to completely inactivate luciferase.

Recently, a functional role in $FMNH_2$ binding has also been indicated for the luciferase β subunit. It was proposed, based on properties of a hybrid consisting of the α from *V. harveyi* luciferase and β from *Photobacterium phosphoreum* enzyme, that both α and β were involved in the initial interaction with $FMNH_2$ whereas subsequent catalytic steps were dictated by the α subunit (Meighen & Bartlet, 1980). Chemical modification of a single amino group on either α or β of the *V. harveyi* luciferase was found to result in the loss of $FMNH_2$-binding ability (Welches & Baldwin, 1981). A study using luciferase immobilized through amino groups on either α or β also indicated that the binding of $FMNH_2$, but not the subsequent catalytic steps, was dependent upon some exposed amino groups on both subunits (Watanabe et al., 1982).

In the present study we have investigated the subunit function of *V. harveyi* luciferase with respect to aldehyde binding. A diazo ketone photoaffinity labeling probe, 1-diazo-2-oxoundecane (DOU), was synthesized and, in the dark, found to be an aldehyde-competitive inhibitor for luciferase. Upon irradiation, covalent attachment of the probe to luciferase occurs. The labeling of either α or β causes inactivation

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[1] Abbreviations: FMN, flavin mononucleotide; $FMNH_2$, reduced FMN; DOU, 1-diazo-2-oxoundecane; [^{14}C]DOU, 1-diazo-2-oxo[1-^{14}C]undecane.

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of the enzyme, indicating that the aldehyde binding site is at the subunit interface of luciferase.

Materials and Methods

General Materials. Bovine serum albumin and FMN were from Sigma. Decanoic acid and decanal were obtained from Eastman and Aldrich, respectively.

Luciferase Purification and Assay. Luciferase was purified from cells of *V. harveyi* as described previously (Hastings et al., 1978). Luciferase activities, in quanta (q) per second, were assayed at 23 °C, by using the standard method (Hastings et al., 1978) of injecting 1 mL of 50 μM $FMNH_2$ into an equal volume of 0.05 M phosphate, pH 7, containing luciferase, 0.1% bovine serum albumin, and 0.001% decanal. Luciferase concentrations were determined based on M_r 79 000 and an absorption coefficient of 1.2 (0.1%, 1 cm) at 280 nm (Tu et al., 1977). The luciferase sample used in the present study was estimated to be >95% pure on the basis of sodium dodecyl sulfate/polyacrylamide gel electrophoresis (Weber & Osborn, 1969) and had a specific activity of 1.9×10^{14} $q \cdot s^{-1} \cdot mg^{-1}$ consistent with that previously reported for homogeneous enzyme (Hastings et al., 1978).

Synthesis of DOU and [^{14}C]DOU. Crystalline decanoic acid (0.265 g; 1.5 mmol) was reacted at 70–75 °C for 1 h in 3 mL of dry benzene containing 0.3 mL of thionyl chloride and 5 mg of dimethylformamide as catalyst. Thin-layer chromatography (silica gel; hexane/ether, 1:1) of an aliquot diluted into methanol visualized by I_2 vapor showed complete conversion to the methyl ester, indicating formation of the acid chloride. The excess solvent and $SOCl_2$ were removed under vacuum with exclusion of moisture, and the oily residue was dissolved in 10 mL of anhydrous ether. This solution was added during 1 min to 10 mL of a stirred ethereal solution of diazomethane (150–200 mg) at 0 °C. After 1 h at 23 °C, the reaction showed a single UV-absorbing spot on the thin-layer chromatogram (silica gel; ether/hexane, 1:1) which bleached under 254-nm irradiation. After removal of solvent under a stream of N_2, the yellow oily residue was taken up in hexane and applied to a column of dry silica gel (8 g, 60–200 mesh). This was flushed with 20 mL of hexane and then eluted with ether/hexane, 1:2. A bright yellow eluting band, when concentrated, gave 0.24 g (80% yield) of pale yellow solid (mp 33 °C). The product was identified as follows: NMR (CCl_4), singlet (δ 5.25, 1 H, diazo), triplets (δ 0.9, 3 H; δ 2.25, 2 H), and complex methylenes (δ 1.1–1.5, 14 H); IR (CCl_4), diazo group (2140 cm^{-1}) and C=O (1650 cm^{-1}); mass spectrum by fast atom bombardment (Barber et al., 1981), single ion at M + 1 (197). Anal. Calcd for $C_{11}H_{20}N_2O$: C, 67.29; H, 10.29; N, 14.27. Found: C, 67.87; H, 10.55; N, 14.44. For the synthesis of [^{14}C]DOU, 50 μCi of *N*-[^{14}C]methyl-*N*-nitroso-*p*-toluenesulfonamide from New England Nuclear was mixed with 20 mg of carrier Diazald from Aldrich in 1 mL of ether and slowly delivered by syringe to a serum-capped side-arm test tube containing ethanolic NaOH at 55 °C. Radioactive diazomethane codistilled with ether through a tube connected to the side arm into an ice-cooled tube. The distillate was used in a reaction identical with that described for the nonlabeled compound, but scaled down by 20-fold. The specific radioactivity of the purified [^{14}C]DOU was 0.35 mCi/mmol.

Photoaffinity Labeling and Quantitation of Probe Incorporation. Photolysis was performed at 23 °C by using a lamp (RPR-2537 from Southern New England UV Co.) with a 254-nm emission peak. Typically, 0.4 mL of 0.05 M NaP_i, pH 7, containing luciferase and DOU or [^{14}C]DOU was added to a cuvette (0.3-cm path length) placed 10 cm from the lamp.

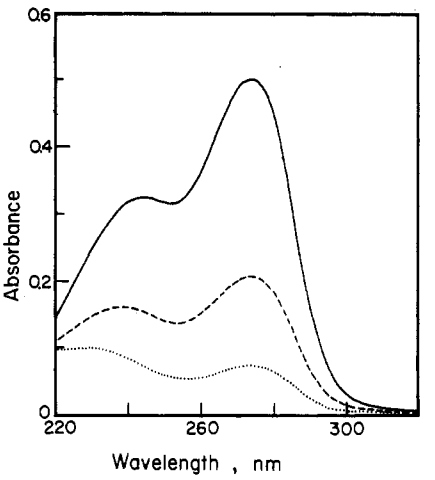


FIGURE 1: Absorption spectra of DOU before and after photolysis. The spectra of DOU, at 65 μM in 0.05 M P_i buffer, pH 7, were measured before (—) and after 30 (- - -) and 60 s (· · ·) of irradiation at 254 nm.

After different times of irradiation, 10-μL aliquots were withdrawn for activity measurements, and 0.1-mL aliquots were each mixed with 2 mg of sodium dodecyl sulfate (from Fisher) and 1 μL of 2-mercaptoethanol (from Eastman). The latter set of aliquots was incubated at 37 °C in the dark for 2 h and then analyzed by sodium dodecyl sulfate/(10%) acrylamide gel electrophoresis (Weber & Osborn, 1969). Gels were stained with Coomassie blue (Weber & Osborn, 1969), destained in methanol/HOAc/H_2O (5:1:5), and washed twice with water (10 mL/gel, 1 h soaking), all at 23 °C. Protein bands were then cut out, sliced into smaller pieces, and solubilized in a capped vial containing 0.5 mL of 30% H_2O_2 for 6–8 h at 65 °C. Subsequently 10 mL of ScintiVerse Universal Cocktail (Fisher) was added to each sample for ^{14}C counting (dpm) in a Beckman LS 9000 liquid scintillation counter.

The amounts of protein in the α and β subunit bands of each gel were based on the quantity of luciferase applied to the gel and a 1:1 molar ratio for the two subunits (Hastings et al., 1969). The amount of ^{14}C probe incorporated into each protein sample was determined based on the known specific radioactivity of [^{14}C]DOU, the measured dpm for each sample, and the overall efficiency of ^{14}C recovery in solubilized gel. For the determination of the overall ^{14}C recovery, a solution containing 11 μM luciferase and 65 μM [^{14}C]DOU was irradiated for 60 s and then extensively dialyzed in the dark to remove free species of both [^{14}C]DOU and photolyzed products derived from it. A 0.1-mL aliquot of the sample was used for ^{14}C counting without further treatment whereas a duplicate aliquot was used for gel electrophoresis followed by staining, destaining, and gel solubilization as described above. The overall efficiency of ^{14}C recovery was calculated to be 93% by a comparison of the total dpm measured for the solubilized α and β subunit bands with that originally applied to the gel.

Results

DOU exhibits absorption peaks at 245 and 275 nm in P_i buffer (Figure 1), with an extinction coefficient of 7.75×10^3 $M^{-1} \cdot cm^{-1}$ at 275 nm. The ratio of A_{245}/A_{275} changes from 0.64 in aqueous solution to 1.12 in ethanol (data not shown), indicating a sensitivity to the medium environment. Upon irradiation, photolysis of DOU occurs as evident from the absorption changes (Figure 1). No spectral alterations were observed over at least several hours when DOU was kept in the dark.

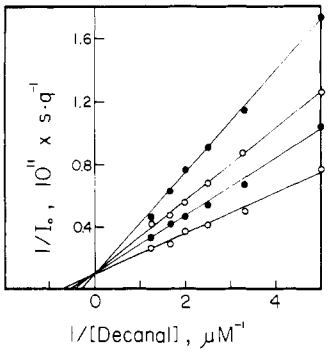


FIGURE 2: DOU as a competitive inhibitor for decanal. The luciferase activity was assayed, by the standard method except that no bovine serum albumin was added, as a function of decanal concentration in the presence of 10 μg of enzyme and, from the bottom line upward, 0.35, 0.58, and 0.81 μM DOU. Results are presented as double-reciprocal plots.

In luciferase assays carried out under dim light, DOU functions as an inhibitor competitive with decanal (Figure 2) with the K_i calculated to be 0.7 ± 0.1 μM. DOU thus appears to specifically and reversibly bind to the aldehyde site of luciferase under such experimental conditions.

The binding of DOU to luciferase was also examined by using a different type of assay. The bacterial bioluminescence reaction involves an oxygenated flavin·luciferase species as a key intermediate (Hastings & Gibson, 1963; Hastings et al., 1973). This intermediate either decays in the absence of aldehyde to produce FMN and H_2O_2 with very little light emission or reacts with aldehyde to yield light and other final products (Hastings & Nealson, 1977). Several types of non-aldehyde long-chain aliphatic compounds have been shown to markedly stabilize this enzyme intermediate by binding, reversibly, to the aldehyde site (Hastings et al., 1966; Baumstark et al., 1979; Tu, 1979). We have found that the oxygenated enzyme intermediate can also be greatly stabilized, at both 0 and 23 °C, by complexing with DOU. The displacement of DOU from the complex by aldehyde is also indicated by the light emission activities observed upon aldehyde addition (Figure 3).

An irreversible inactivation of luciferase occurred, following apparent first-order kinetics, when the enzyme was irradiated in the presence of DOU (Figure 4). This inactivation was not observed when the sample was shielded from light and was retarded ($\simeq$2-fold) by the addition of decanal ([decanal]/[DOU] = 7.6). The addition of 2-mercaptoethanol as a ketene scavenger (see Discussion) also decreased the photoinactivation rate. Since 2-mercaptoethanol (at 13 mM in a 0.3-cm cuvette) absorbs significantly in the emission range of the lamp (e.g., absorption of 32% of the light at 245 nm), the slower photoinactivation observed is, at least in part, attributed to a reduced flux of light reaching DOU. Only low levels of photoinactivation were detected when luciferase was irradiated either alone or in the presence of decanal.

Irradiation of [^{14}C]DOU and luciferase also resulted in apparent covalent attachment of the probe to both α and β subunits (Figure 5A). Up to about 60% inactivation (corresponding to 0.64 nmol of enzyme inactivated per 0.1 mL of sample after 30 s of photolysis), the rate of probe incorporation into α was about the same as that for β. However, larger amounts of the probe were attached to the α subunit than to β at higher levels of inactivation, reaching approximately a 2-fold difference after 60 s of photolysis (corresponding to $\simeq$0.9 nmol of enzyme inactivated per 0.1 mL of sample). The labeling of α or β alone was insufficient to quantitatively

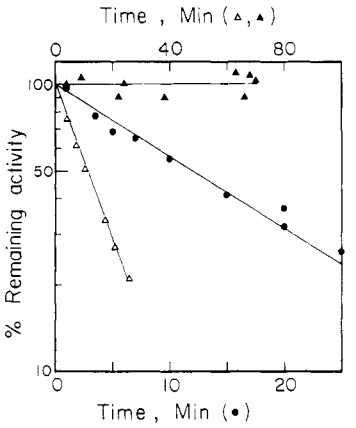


FIGURE 3: Effect of DOU on the stability of oxygenated flavin·luciferase intermediate. Two samples of the oxygenated enzyme intermediate (△, ▲) were each formed at 0 °C by mixing 0.5 mL of an air-saturated 0.05 M P_i solution, pH 7, containing 50 μg of luciferase with an equal volume of the same buffer containing 50 μM $FMNH_2$ (reduced catalytically with H_2). To one sample (▲) was quickly added 50 μL of 1.2 mM DOU in 95% ethanol to form the intermediate·DOU complex. Both samples were kept at 0 °C in the dark. Aliquots (50 μL each) were withdrawn after different times and each injected into 1 mL of 0.05 M P_i, pH 7, containing 0.005% decanal at 23 °C for bioluminescence activity measurements. The rate of decrease in the remaining bioluminescence capacity provides a measure of the stability of the intermediate. The decay rates of the intermediate at 23 °C were similarly determined in the presence (●) and absence of DOU. Under the latter condition, the intermediate rapidly decayed with a half-life of about 9 s (data not shown).

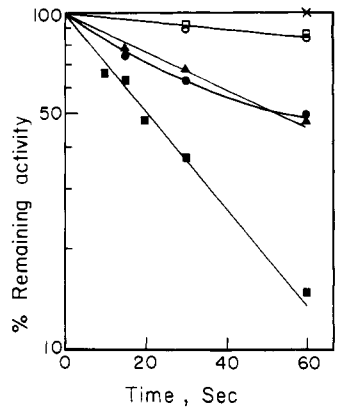


FIGURE 4: Kinetics of luciferase photoinactivation. A 0.4-mL 0.05 M P_i solution, pH 7, containing 10.7 μM luciferase and 65 μM [^{14}C]DOU was irradiated as described under Materials and Methods. Aliquots (10 μL each) were withdrawn after different times for measurements of remaining enzyme activities (■). Similar experiments were carried out by using samples containing, in addition to luciferase and [^{14}C]DOU as specified above, 0.5 mM decanal (●) or 13 mM 2-mercaptoethanol (▲). As controls, the activities of samples containing just the enzyme (□) or enzyme plus 0.5 mM decanal (○) were also measured after different times of irradiation. In another control, the activity of a sample containing luciferase and DOU was measured after 60 s of incubation in the dark (×).

account for the extent of luciferase inactivation whereas a molar ratio of 1.4 (correlation coefficient 0.95) for total probe incorporated into α and β to enzyme inactivated was observed throughout the entire 60 s of photolysis (Figure 5A). Photolabeling of both α and β subunits by [^{14}C]DOU also occurred in the presence of decanal (Figure 5B) or 2-mercaptoethanol (Figure 5C). In neither case can enzyme inactivation be solely attributed to the modification of α or β alone. The molar ratios of probe incorporated into α and β to enzyme inactivated were 1.3 and 1.0 (respective correlation coefficients 0.97 and 0.96) for the decanal- and 2-mercaptoethanol-containing samples, respectively.

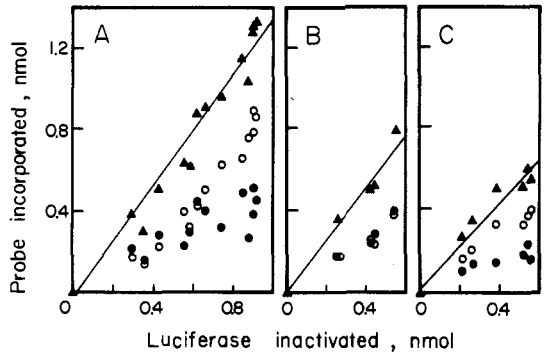
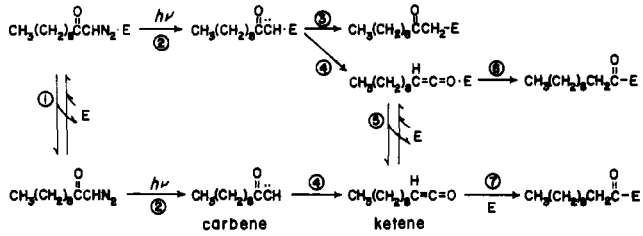


FIGURE 5: Correlation of the amount of luciferase photoinactivated with that of probe incorporated. In the experiments described in Figure 4, 100-μL aliquots were also withdrawn from samples containing luciferase and [^{14}C]DOU with either no other additions (A), 0.5 mM decanal (B), or 13 mM 2-mercaptoethanol (C) after different times of photolysis for the determination of the amounts of probe covalently attached to individual subunits as described under Materials and Methods. The quantity of probe incorporated into α (○), β (●), or α and β subunits (▲) is plotted as a function of the amount of luciferase photoinactivated, all expressed in nmol/100 μL of sample which contained 1.07 nmol of the dimeric enzyme. The value of cpm measured for a solubilized gel blank was 15, and that for a solubilized gel sample containing 0.1 nmol of ^{14}C probe incorporated into protein (the smallest amount of ^{14}C incorporation in all the samples) was 35 above the blank. All samples were counted for 10 min. The linear lines shown were fitted by least-squares analysis, and the slopes are 1.4 (A), 1.3 (B), and 1.0 (C).

Scheme I



Discussion

Successful applications of (photo)affinity labeling techniques for the elucidation of bacterial luciferase active site structure have not been previously reported. In the present investigation, we have synthesized DOU as a photoaffinity probe for the luciferase aldehyde-binding site, and this aliphatic diazo ketone appears to satisfy the criteria conventionally designated for such an agent. These include (a) chemical stability in the dark allowing preequilibrium with enzyme for the formation of a noncovalent complex, (b) specific binding to active site competitive with the decanal substrate (Figures 2 and 3), (c) generation of active species upon photolysis leading to inactivation and labeling of enzyme (Figures 4 and 5), and (d) protection by substrate (decanal) against photoinactivation and labeling (Figures 4 and 5). However, photolabeling of luciferase by DOU proceeds by both specific and nonspecific pathways as depicted in Scheme I. Upon photolysis (step 2), both free DOU and bound DOU are activated to generate the highly reactive carbene species. The bound carbene may then specifically tag amino acid residue(s) at the active center (step 3). Since carbenes are probably very short-lived in water (Moss, 1973), nonspecific labelings due to collisions of enzyme molecules with free carbenes are probably insignificant. However, both free and bound carbene can undergo the Wolff rearrangement (Wolff, 1912; Kirmse, 1971) to form the electrophilic ketene (step 4). While the bound ketene may label the active center specifically (step 6), the free ketene derived either from the free carbene or by dissociation from the bound species (step 5) is, due to its longer lifetime (Kirmse, 1971), potentially capable of reacting with certain enzyme nucleophilic groups not located at the active center (step 7).

We have found that both α and β subunits of luciferase can be photolabeled by DOU. However, while the modification of α or β alone could not account for the degree of luciferase inactivation, the total molar quantities of probe incorporated into α and β exceeded that of the enzyme inactivated by 40% (Figure 5A), indicating the existence of significant nonspecific labeling of α and/or β by free ketenes. Similar photolysis experiments were also carried out in the presence of decanal to specifically protect the active center and 2-mercaptoethanol, a ketene scavenger, to quench the nonspecific modification (Bayley & Knowles, 1977; Chowdhry & Westheimer, 1979). Decanal retarded both the rates of enzyme inactivation and photolabeling of α and β. However, at levels up to 60% enzyme inactivation, the molar ratio of probe incorporation into $\alpha\beta$ over enzyme inactivated remained at about 1.3 (Figure 5B), and the quantities of probe attached to α and β were each about the same as that observed with the aldehyde-free sample at corresponding degrees of inactivation (Figure 5A,B). Therefore, the addition of decanal was not expected to and apparently did not prevent the nonspecific labeling. It should be noted that the extent of nonspecific labeling is expected to be somewhat greater in the aldehyde-containing sample than in the aldehyde-free sample at the same level of photoinactivation. Our results did not show such a difference between the two samples in this regard (Figure 5A,B), and the reason for this is, at the present moment, unclear. On the other hand, the degrees of photolabeling of α in the 2-mercaptoethanol-containing sample, at levels up to about 60% enzyme inactivation, were quite comparable to those shown in Figure 5A,B, but significantly smaller amounts of probe tagged the β subunit (Figure 5C). Most importantly, the total amounts of probe incorporated into $\alpha\beta$ in the 2-mercaptoethanol-containing sample correlated stoichiometrically with the quantities of enzyme inactivated, indicating that the labelings of both α and β were, in this case, primarily via the specific pathways (Scheme I).

We interpret these results by postulating that the aldehyde binding site is located at the subunit interface of luciferase. The photoactivated probe generated in situ at the active center may covalently tag either subunit following the specific mechanisms (Scheme I), thus preventing further bindings of DOU or aldehyde to the active site. Consequently, the specific labeling of α and that of β are mutually exclusive, and each is a killing event with respect to enzyme activity. However, in most experiments, free ketenes apparently also lead to a lower but significant level (~40%) of nonspecific labeling of luciferase, primarily on the β subunit.

Added in Proof

An account of this work was recently presented at the 38th Southwest Regional Meeting of the American Chemical Society (Tu & Henkin, 1982). At the same meeting a model, based on binding of aldehyde to wild-type and mutant luciferases, was also presented which suggests that the aldehyde binding site resides at the luciferase subunit interface (Holzman & Baldwin, 1982), consistent with our finding.

Registry No. DOU, 76917-15-2; [^{14}C]DOU, 83897-73-8; decanoic acid, 334-48-5; decanal, 112-31-2; luciferase, 9014-00-0.

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