Active Center Studies on Bacterial Luciferase: Modification of the Enzyme with 2,4-Dinitrofluorobenzene†

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ABSTRACT: Bacterial luciferase catalyzes the mixed-function oxidation of a long-chain saturated aldehyde and $FMNH_2$ to yield the carboxylic acid, FMN, and blue-green light. The enzyme was inactivated by 2,4-dinitrofluorobenzene (FDNB) with an observed second-order rate constant ($k_{2(obsd)}$) of 157 $M^{-1}\cdot min^{-1}$ at pH 7.0, 25 °C; activity was not recovered upon treatment with 2-mercaptoethanol (thiolysis), demonstrating that the inactivation was the result of reaction with one or more amino groups. The dinitrophenyl (DNP) moiety was incorporated into the α subunit approximately twice as fast as it was incorporated into the β subunit; the rate of inactivation was nearly identical with the rate of incorporation into the $\alpha\beta$ dimer. The incorporation of 1 mol of DNP/$\alpha\beta$ resulted in complete inactivation, demonstrating that modification of either α or β is sufficient to cause inactivation. Incorporation of DNP into one subunit appeared to either block or decrease the rate of incorporation of DNP into the other subunit. The luciferase was protected from inactivation by binding of long-chain aldehydes or FMN. Following modification by FDNB, the enzyme had lost measurable $FMNH_2$ binding. The apparent pK_a of the amino groups, determined by analysis of the pH dependence of the inactivation reaction, was 9.4. This value is too high to allow correlation with the pH–activity profile of the enzyme [Nicoli, M. Z., Meighen, E. A., & Hastings, J. W. (1974) *J. Biol. Chem. 249*, 2385–2392]. The catalytic function, if any, for the reactive amino groups remains unknown.

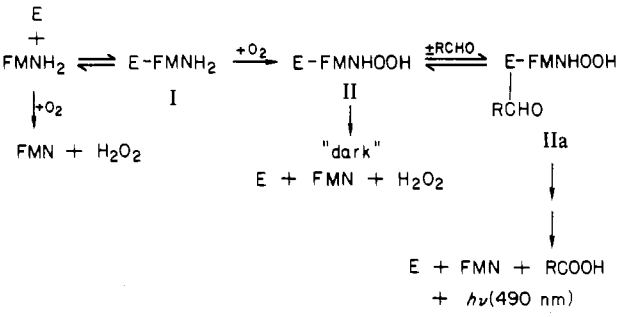
Bacterial luciferase is a flavin monooxygenase responsible for light emission in luminous marine bacteria. The major features of the reaction catalyzed by this enzyme are shown in Scheme I (Hastings & Gibson, 1963; Dunn et al., 1973; Shimomura et al., 1972; McCapra & Hysert, 1973; Vigny & Michelson, 1974).

The enzyme is composed of two nonidentical subunits, α and β, with molecular weights of 42 000 and 37 000, respectively (Hastings et al., 1969). Extensive chemical modification (Meighen et al., 1971a,b; Nicoli et al., 1974; Nicoli & Hastings, 1974) and mutant enzyme analyses (Cline & Hastings, 1972) have shown that the active center of the enzyme resides primarily, if not exclusively, on the α subunit. The precise role of the β subunit is unclear, although it is required for activity (Friedland & Hastings, 1967; Gunsalus-Miguel et al., 1972).

Detailed analysis of the reaction of alkylating reagents with the luciferase from *Beneckea harveyi* has demonstrated the existence of a highly reactive cysteinyl residue on the α subunit (Nicoli et al., 1974). Modification of this cysteinyl residue resulted in loss of enzymatic activity and loss of measurable $FMNH_2$[1] binding. The observed rate of modification of the cysteinyl residue by alkylating reagents was greatly decreased in the presence of either FMN or long-chain aldehydes. Kosower (1980) has proposed a role for a cysteinyl residue in the bioluminescence reaction. Cousineau & Meighen (1976) have shown that modification of a histidyl residue with ethoxyformic anhydride at pH 6.0 results in loss of activity. This histidyl residue was protected from modification by long-chain aldehydes.

It appears likely that both the cysteinyl residue and the histidyl residue reside in or near the active center of the enzyme. The experiments reported here were undertaken to supply more detailed information about the chemistry of the active center of luciferase. The reagent chosen for this investigation was 2,4-dinitrofluorobenzene (FDNB), which will react with primary amines and the functional groups of cysteinyl, histidyl, and tyrosyl residues (Sanger, 1945; Porter & Sanger, 1948). The DNP adducts with amino groups (α or ϵ) are relatively stable, but the DNP adducts of cysteinyl, histidyl, and tyrosyl residues are readily reversible by thiolysis (Shaltiel, 1967). The previous studies on alkylation of luciferase (Nicoli et al., 1974) suggested that FDNB might react preferentially with the reactive cysteinyl residue on the α subunit. This adduct should be readily reversible by thiolytic cleavage with 2-mercaptoethanol or dithioerythritol. Our results demonstrate clearly that FDNB does *not* react with the reactive cysteinyl residue; it reacts first with an amino group on the α subunit and, at a slower rate, with an amino group on the β subunit. Modification of either group leads to inactivation of the enzyme.

Scheme I



Experimental Procedure

Materials. The DNP amino acid standards, the N^{α}-acetylated lysine and cysteine, and the methionine were ob-

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[1] Abbreviations used: FMN, riboflavin 5′-phosphate; $FMNH_2$, reduced FMN; FDNB, 2,4-dinitrofluorobenzene (Sanger's reagent); DNP, 2,4-dinitrophenyl; $NaDodSO_4$, sodium dodecyl sulfate; MMTS, methyl methanethiosulfonate; DTE, dithioerythritol.

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tained from Sigma. The long-chain aldehydes, MMTS, and DTE were products of Aldrich. The FDNB was obtained from Pierce Chemical Co. and [3,5-^{3}H]FDNB from New England Nuclear. FMN was a gift from Sigma. All other compounds were of reagent quality or better and were used without further purification. Phosphate buffers were prepared by dilution of appropriate volumes of 2 M stock solutions of K_2HPO_4 and NaH_2PO_4. Pyrophosphate buffers were prepared by adjusting the pH of solutions of the sodium salt with HCl and diluting to 0.05 M.

Luciferase Purification and Assay. The *Beneckea harveyi* cells were grown, and the luciferase was purified as described (Hastings et al., 1978). Following chromatography on aminohexyl-Sepharose 6B, the purity of the enzyme, estimated from Coomassie Blue staining of $NaDodSO_4$ gel (Laemmli, 1970), was greater than 95%. Luciferase samples were stored frozen in phosphate buffer with DTE. The luciferase activity was measured by the standard $FMNH_2$ injection assay (Hastings et al., 1978); light emission was monitored by using a photomultiplier photometer (Mitchell & Hastings, 1971).

Concentration Determinations. The concentration of luciferase was based upon an abosrption coefficient at 280 nm of 0.94 mL·mg^{-1}·cm^{-1} and a molecular weight of 79 000 (Gunsalus-Miguel et al., 1972). The contribution of the DNP group to the absorbance of the modified protein (about 6% increase) was ignored, except in the $FMNH_2$ binding measurements. In these experiments, the luciferase concentration was determined by the method of Lowry et al. (1951).

The concentrations of the various DNP derivatives were determined by using the following molar extinction coefficients: DNP-2-mercaptoethanol, ϵ_{330} = 12 250 M^{-1}·cm^{-1}; *S*-DNP-cysteine, ϵ_{330} = 10 500 M^{-1}·cm^{-1}; N^{ϵ}-DNP-lysine, ϵ_{360} = 17 700 M^{-1}·cm^{-1} (Kowal et al., 1965).

Conditions of Luciferase Inactivation. Luciferase was exhaustively dialyzed against phosphate buffer, pH 7.0, to remove the reducing agent in the storage buffer. Enzyme samples were equilibrated at 25 °C in an electronic temperature controller before addition of FDNB. Reactions were initiated by addition of an appropriate volume of 79 mM FDNB prepared in ethanol. The concentrations of reagent and enzyme were such that pseudo-first-order conditions were maintained for the duration of the reaction. The inactivation of the luciferase was monitroed by assay of aliquots at various times for bioluminescence activity. The reactions were effectively quenched by 1:100 dilution of the reaction mixture into an assay buffer containing decanal; activities were measured immediately after dilution.

In experiments with [3,5-^{3}H]FDNB, the reactions were initiated by addition of enzyme to the reagent, both at 25 °C. Reactions were stopped either by addition of excess 2-mercaptoethanol or by chilling the reaction mixture to 0 °C and column chromatography on Sephadex G-25. In order to retain a sufficiently high specific radioactivity, pseudo-first-order conditions were not always possible in these experiments. Radioactivity in the luciferase subunits was detected by fluorographic analysis of $NaDodSO_4$–polyacrylamide slab gels (Bonner & Lasky, 1974; Lasky & Mills, 1975).

$FMNH_2$-Binding Experiments. The stoichiometry of binding of $FMNH_2$ to native and modified luciferases was measured by using the kinetic technique of Meighen & Hastings (1971). In this method, intermediate I (see Scheme I) is generated in an anaerobic solution by addition of sodium dithionite. The bioluminescence reaction is then initiated by injection of an air-equilibrated aldehyde suspension. The light emission is proportional to the initial concentration of intermediate I. The method allows determination of the concentration of $FMNH_2$ binding sites in solution.

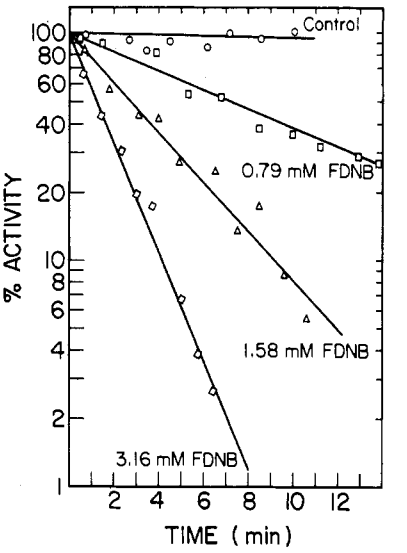

FIGURE 1: Kinetics of luciferase inactivation by FDNB. Reactions were initiated by addition of FDNB (79 mM, in ethanol) to luciferase (final concentration 38 μM) in 0.05 M phosphate, pH 7.0, at 25 °C. Aliquots were withdrawn and diluted 1:100 into assay buffer at the designated times. Luciferase activity was determined immediately and recorded as percent of the initial value. control (○), 0.79 mM FDNB (□), 1.58 mM FDNB (△), 3.16 mM FDNB (◇).

Ultracentrifugation. The sedimentation behavior of native and modified luciferase samples was measured with a Beckman Model E analytical ultracentrifuge. Samples were sedimented at 40 000 rpm in an AN-F rotor at 20 °C. The progress of the sedimentation was monitored with a photoelectric scanner.

Reaction of FDNB with Model Compounds. The rates of reaction of FDNB with 2-mercaptoethanol, N^{α}-acetylated lysine and cysteine, and methionine were determined at 25 °C in 0.02 M phosphate buffer, pH 7.0. The reactions were monitored at the appropriate wavelengths with a Cary 15 spectrophotometer.

Results

Rate of Inactivation. The addition of FDNB to solutions of luciferase in 0.05 M phosphate, pH 7.0, at 25 °C results in a rapid inactivation of the luciferase (Figure 1). In the experiment depicted in Figure 1, the FDNB was always in such excess that pseudo-first-order conditions were maintained for the duration of the experiments. That is, the concentration of FDNB was essentially constant throughout each experiment, and the rate of inactivation was dependent on the concentration of FDNB but independent of luciferase concentration. The apparent second-order rate constant for the inactivation of luciferase by FDNB was determined from these data to be 157 M^{-1}·min^{-1}.

Protection of the Reactive Thiol with Methyl Methanethiolsulfonate (MMTS). Treatment of the luciferase with a 2-fold molar excess of MMTS resulted in a rapid inactivation of the enzyme which was totally reversible by treatment with 2-mercaptoethanol[2] (Figure 2). After the reaction of MMTS with luciferase was complete, a very slight spontaneous reactivation occurred, perhaps due to disulfide exchange. Addition of FDNB to the MMTS-modified luciferase (about 1% residual activity) caused further inactivation. After 90% of the luciferase activity that existed at the time of addition of FDNB had been lost, the reaction was quenched, and the

[2] M. Ziegler (Nicoli), unpublished results.

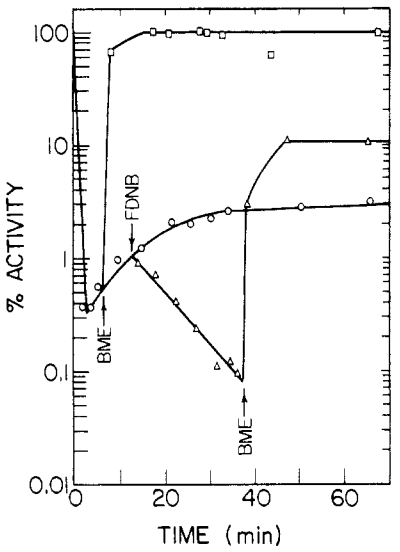

FIGURE 2: Protection of the reactive thiol by reaction with methyl methanethiolsulfonate. Luciferase (68 μM) was allowed to react with a 2-fold molar excess of MMTS at 25 °C in 0.02 M phosphate, pH 7.0. Three aliquots of the modified protein were taken. The control aliquot (○) was monitored for luciferase activity. The thiolysis aliquot (□) was treated with 2-mercaptoethanol (97 mM) at the time indicated. The FDNB inactivation aliquot (△) was allowed to react with FDNB (1.93 mM) until 90% of the luciferase activity measured at the time of FDNB addition had been lost. At this time, 2-mercaptoethanol (97 mM) was added, and the luciferase activity was monitored for an additional 30 min.

SCH_3 blocking group was removed by addition of 2-mercaptoethanol. The thiolysis resulted in recovery of 10% of the initial activity rather than 100%, demonstrating that the luciferase activity is sensitive to FDNB, even if the reactive thiol is not available for reaction. The results of this experiment demonstrate that the inactivation of luciferase by FDNB is not due to reaction of the same thiol that reacts with MMTS, iodoacetamide, and other alkylating reagents.

Stoichiometry of the Reaction. The stoichiometry of the reaction of FDNB with luciferase was determined by using [3,5-^{3}H]FDNB. Luciferase was allowed to react with a 2.8-fold molar excess of FDNB for 3 h. The sample was then divided into two aliquots, a and b. Sample a was dialyzed overnight at 4 °C against 0.05 M phosphate buffer, pH 7.0, and sample b was dialyzed against the same buffer with 5 mM 2-mercaptoethanol. Both samples were then subjected to column chromatography on Sephadex G-25 to remove residual traces of noncovalently bound reagent. Radioactivity, protein, and luciferase activity were monitored. Both samples had less than 1% of the initial luciferase activity. Sample a had 1.0 mol of DNP/mol of luciferase, and sample b had 0.9 mol of DNP/mol of luciferase.

These observations demonstrate that inactivation of luciferase by FDNB is the result of modification of an amino group to form a species that is not susceptible to thiolysis (Shaltiel, 1967). The possible sites of modification are the α-amino groups and the ϵ-amino groups. The NH_2-terminal residue of both subunits is methionine (Baldwin et al., 1979). For the determination of the identity of the residue modified by FDNB, the acid hydrolysis–ether extraction method was used (Sanger, 1945; Porter & Sanger, 1948).

All experiments resulted in a variety of products in the acid phase that could not be correlated by electrophoresis on paper at pH 1.9 with ϵ-DNP-lysine, *S*-DNP-cysteine, *O*-DNP-tyrosine or *im*-DNP-histidine. On the other hand, only very small quantities ($\leq$10%) of the product which was extracted into ether cochromatographed with α-DNP-methionine. ϵ-DNP-lysine is quite stable and should be readily identifiable in the acid phase. However, α-DNP amino acids are both acid labile and photolabile, and α-DNP-methionine is one of the least stable DNP amino acids (Porter & Sanger, 1948). For detection and quantification of the modified residue, great care was taken to exclude light and minimize hydrolysis times, but these efforts were frustrated by the instability of the initial product. In spite of our inability to unambiguously isolate and identify the residue modified, our observations suggest that the inactivation of luciferase by FDNB is most likely due to reaction with the α-amino group of the NH_2-terminal methionine of the α and/or β subunit.

It is possible that the inactivation of luciferase by reaction with FDNB is due to protein denaturation rather than to modification of an essential group. The sedimentation behavior of luciferase modified with FDNB was compared with that of native luciferase in the ultracentrifuge to test this possibility. The two samples behaved identically, demonstrating that modification by FDNB does not result in subunit dissociation or protein aggregation.

Effect of Modification on $FMNH_2$ Binding. The inactivation of the luciferase that accompanies incorporation of a single DNP group could be due to a decrease in affinity of the enzyme for $FMNH_2$ and/or aldehyde, blocking of a step in the reaction (Scheme I), or quenching of the excited state of the emitter. To determine the reason for the inactivation, we measured the binding of $FMNH_2$, which is the first step in the reaction sequence (Scheme I). The method used is the kinetic technique described by Meighen & Hastings (1971). This approach allows determination of the concentration of $FMNH_2$ binding sites in solution. The results of our measurements demonstrate that modification of the enzyme with FDNB causes a loss of measurable binding of $FMNH_2$ (data not shown). When 71% of the luciferase activity was lost, 68% of the $FMNH_2$ binding sites were lost. If the inactive enzyme binds $FMNH_2$, the dissociation constant is at least 10 times the dissociation constant of the $FMNH_2$–native luciferase complex (Nicoli et al., 1974). It is clear that the primary reason for the loss of luciferase activity is the loss of $FMNH_2$ binding.

Protection by Substrate and Product. The fact that incorporation of a single mole of DNP per mole of luciferase resulted in inactivation of the enzyme and loss of measurable $FMNH_2$ binding suggested that the modification causes a structural perturbation in the active center. Therefore, the effects of aldehyde binding and of FMN binding upon the rate of inactivation of luciferase by FDNB were determined. Longer chain-length aldehydes afforded greater protection than shorter chain-length aldehydes (Figure 3). Nicoli et al. (1974) made a comparable observation in their experiments with sulfhydryl modification.

The binding of FMN resulted in a dramatic decrease in the sensitivity of luciferase to inactivation by FDNB (Figure 5). The concentration of FMN used (32 mM) was 75 times the FMN dissociation constant at 25 °C (about 0.42 mM; Baldwin, 1974; Baldwin et al., 1975), and the observed rate of inactivation by FDNB (21 $M^{-1}\cdot min^{-1}$) was about one-tenth the rate of inactivation in the absence of FMN.

Determination of the pK_a of the Reactive Group. For a second-order reaction of an alkylating reagent, such as FDNB, with an ionizing nucleophile, such as an amino group, the observed second-order rate constant, $k_{2(obsd)}$, will reflect the fraction of the nucleophile in the unprotonated form. With the assumption that the rate of alkylation of the protonated nucleophile is negligible compared with the rate of alkylation

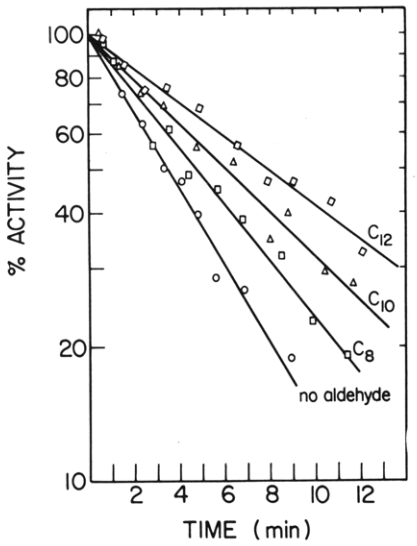

FIGURE 3: Protection of luciferase by aldehyde. Luciferase at 25 °C (final concentration 38 μM, in 0.05 M phosphate, pH 7.0) was allowed to equilibrate for 30 s with 0.001% (v/v) sonicated aldehyde suspension. Reactions were initiated by addition of FDNB to 2.0 mM [no aldehyde (○), octanol (□), decanal (△) dodecanal (◇)].

of the unprotonated nucleophile, the relation between $k_{2(obsd)}$, $\bar{k}_2$, the absolute second-order rate constant, and pK', the apparent pK_a of the amino group, is given by

$$k_{2(obsd)} = \frac{\bar{k}_2 K'}{K' + [H^+]} \quad (1)$$

which represents the reaction

$$E\text{-}NH_3^+ \rightleftharpoons^{K'} E\text{-}NH_2 \xrightarrow{\bar{k}_2} E\text{-}N(H)\text{-}DNP \quad (2)$$

(FDNB; H^+)

Equation 1 may be written as

$$\frac{1}{k_{2(obsd)}} = \frac{1}{\bar{k}_2} + \frac{[H^+]}{K'\bar{k}_2} \quad (3)$$

A plot of $1/k_{2(obsd)}$ vs. $[H^+]$ should give a straight line with an ordinate intercept of $1/\bar{k}_2$ and a slope equal to $1/K'\bar{k}_2$. Such a plot of the data in Figure 4 is shown in the inset to Figure 4. The ordinate intercept of this plot indicates that the absolute second-order rate constant is 2.4×10^5 $M^{-1}\cdot min^{-1}$, 3 orders of magnitude higher than the observed rate at pH 7.0 (157 $M^{-1}\cdot min^{-1}$). The slope of the line indicates an apparent pK_a of 9.4. This value is lower than expected for an ϵ-amino group, but is within the range expected for an α-amino group.

Subunit Localization of the Reactive Group. For determination of the subunit localization of the DNP group which causes inactivation, an experiment was performed with [3,5-^{3}H]FDNB under conditions that resulted in incorporation of about 1 mol of DNP/mol of luciferase and essentially total inactivation. Luciferase was allowed to react with a 2-fold molar excess of FDNB, and the time course of the inactivation was monitored. At various times, aliquots were withdrawn and diluted into a $NaDodSO_4$ sample buffer containing 2-mercaptoethanol (Laemmli, 1970). These samples were subjected to $NaDodSO_4$–slab gel electrophoresis, and the location of the radioactive material was determined by fluorography (Bonner & Laskey, 1974; Laskey & Mills, 1975) (Figure 5). The X-ray film was scanned with an Ortec Model 4310 densitometer and the total amount of radioactivity determined from a standard curve generated by using polyacrylamide slabs containing known amounts of [^{3}H]leucine;

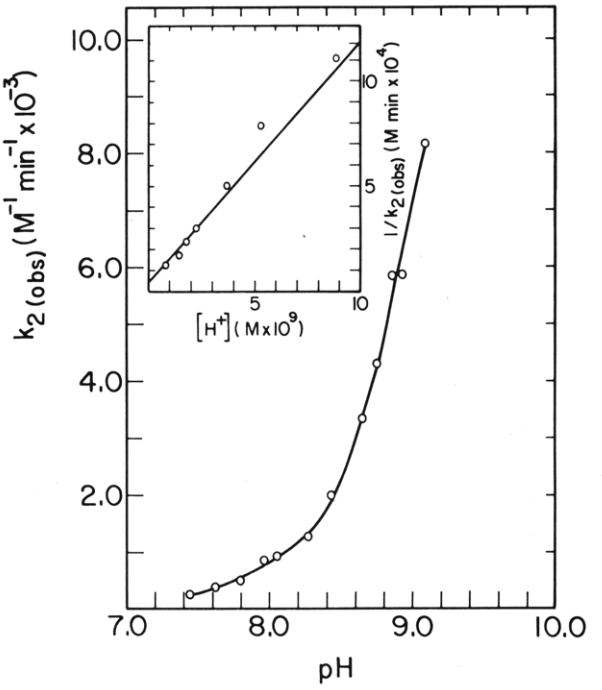

FIGURE 4: pH dependence of the rate of activation. Luciferase (1.1 μM) was allowed to react with FDNB at 25 °C in 0.05 M pyrophosphate buffer that had been adjusted to various values of pH. The apparent second-order rate constant was determined at each pH. Since the luciferase is unstable above pH 9.5, the entire titration curve could not be determined; rather, the data were plotted in a linear form (inset) to allow evaluation of the pK_a and $\bar{k}_2$, the second-order rate constant for reaction of the unprotonated group (see text).

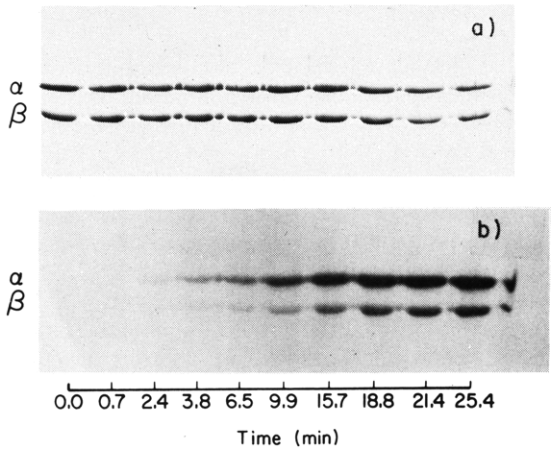

FIGURE 5: Subunit localization of the sites of reaction. Luciferase (130 μM) was allowed to react with a 2-fold molar excess of [3,5-^{3}H]FDNB. Luciferase activity was monitored by the $FMNH_2$ injection assay (Figure 6), and aliquots were withdrawn at various times and diluted 1:2 into a 2-fold concentrated $NaDodSO_4$ gel sample buffer (Laemmli, 1970). The samples were subjected to electrophoresis in 15% polyacrylamide gels as described (Laemmli, 1970). The protein was stained with Coomassie Brilliant Blue (R-250) and photographed (Panel a). The gel was then impregnated with 2,5-diphenyloxazole (PPO) in dimethyl sulfoxide. The gel was dried on Whatman 3MM paper and placed against Kodak XRP-5 X-ray film for 5 days at −70 °C (Bonner & Laskey, 1974). The X-ray film was developed and photographed (panel b).

a plot of the peak areas obtained from the densitometer scans is presented in Figure 6.

It is evident in Figure 5 that both subunits are being modified by FDNB. The incorporation of label into the α subunit appears to proceed at a higher rate than incorporation of label into the β subunit. A quantitative representation of these observations is presented in Figure 6. The loss of luciferase activity proceeded significantly faster than the in-

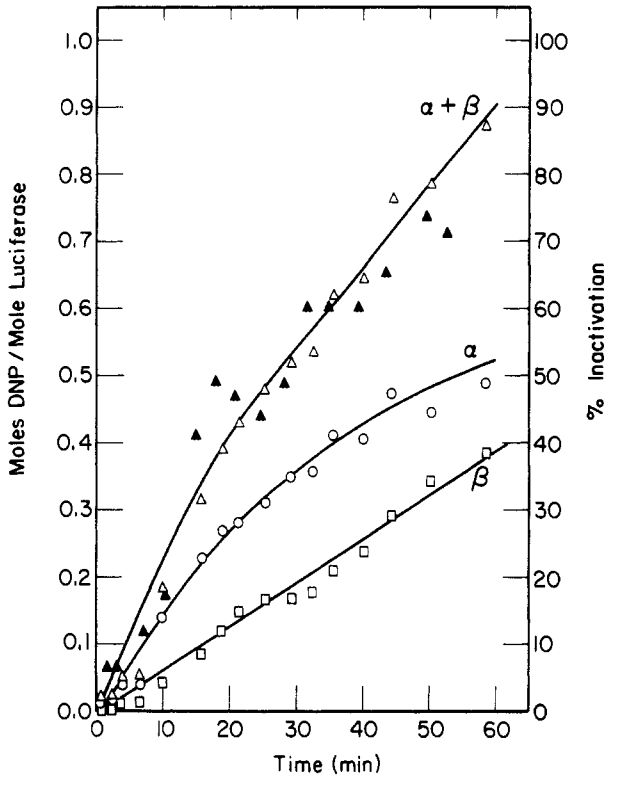

FIGURE 6: Kinetics of subunit modification. The amount of DNP per subunit was measured by integration of peaks obtained by scanning the X-ray film depicted in panel b of Figure 5 with an Ortec Model 4310 densitometer. The amount of DNP on the α subunit (○), and β subunit (□) and the sum of α and β (△) are compared with the percent inactivation (▲).

corporation of label into either α or β and more closely approximates the rate of incorporation into total luciferase, i.e., $\alpha + \beta$. When 60% of the luciferase activity had been lost, there was about 0.4 mol of DNP/mol of α and 0.2 mol of DNP/mol of β, indicating that modification of β can cause inactivation.

Reaction of FDNB with Model Compounds. For determination of the significance of the observed second-order rate constant for inactivation of luciferase by FDNB, 157 $M^{-1} \cdot min^{-1}$, the rate constant for reaction of FDNB with various small molecules was determined by observing the rate of appearance of yellow color with the Cary 15 spectrophotometer. The results are presented in Table I. It is clear from these data that the rate of inactivation of luciferase by FDNB was much faster at pH 7.0, 25 °C, than the reaction of FDNB with *N*-acetylcysteine, the most reactive of the compounds analyzed. The reaction of the α-amino group of methionine with FDNB was about 5 times faster than the reaction of the ϵ-amino group of lysine with FDNB, probably reflecting the lower pK_a of the α-amino group. However, the rate of inactivation of luciferase by FDNB, apparently due to reaction with amino groups of both subunits, was about 2000 times faster than the rate of reaction of FDNB with methionine and more than 10 000 times the rate of reaction of FDNB with the ϵ-amino group of lysine.

Table I: Apparent Second-order Rate Constants for Reaction of Functional Groups with 2,4-Dinitrofluorobenzene[a]

reagent or protein	functional group	$\bar{k}_2(obsd)$ ($M^{-1} \cdot min^{-1}$)
2-mercaptoethanol[a]	SH	5.5
N-acetylcysteine[a]	SH	6.3
N^{α}-acetyllysine[a]	NH_2	0.013
methionine[a]	NH_2	0.070
luciferase[b]	NH_2	157

[a] Reactions were performed at pH 7.0, 20 °C, in 0.02 M phosphate buffer; the progress of the reaction was monitored spectrophotometrically. [b] The apparent second-order rate constant for reaction with luciferase was derived from the data in Figure 1.

Discussion

The results of our experiments demonstrate clearly that bacterial luciferase is inactivated by reaction with FDNB. The reagent appears to react preferentially with amino groups of both the α and the β subunits. Modification of either will cause inactivation, and reaction appears to be mutually exclusive. That is, it appears that incorporation of label into one subunit either blocks or decreases the rate of incorporation into the other subunit. A comparable "mutually exclusive reaction" has been reported for alkylation of the active-center histidyl residues of ribonuclease (Gundlach et al., 1959; Crestfield et al., 1963a,b).

The pH dependence of the inactivation demonstrates an apparent pK_a of 9.4, too high to allow correlation with the pH–activity profile of the enzyme (Nicoli et al., 1974). Clearly, the preferential modification of the amino groups cannot be due to a lower pK_a since the rate of inactivation is 2000 times faster than the rate of reaction of FDNB with methionine (Table I), which has a pK_a of 9.21 (Emerson et al., 1931), and over 10 000 times faster than the rate of reaction of FDNB with the ϵ-amino of lysine (Table I), which has a pK_a of 10.7 (Dawson et al., 1969). Furthermore, the preferential reactivity cannot simply reflect accessibility since the free amino acid should be more exposed for collision with the reagent than the residue in the protein.

To distinguish between an affinity binding effect and an increased nucleophilicity caused by interaction with other amino acid residues in the protein is not easy. In general, there is a direct relationship between nucleophilicity and basicity (Jencks, 1969). However, since the apparent pK_a is 9.4, it would appear unlikely that the enhanced reactivity could be due to enhanced nucleophilicity. It appears more likely that the dramatic rate enhancement would be due to an affinity binding effect resulting in proper orientation of the reagent for reaction with the amino group.

Many enzymes have been reported to have "essential" amino acid residues, i.e., to be inactivated as a consequence of modification of a specific residue. Such an observation by no means demonstrates that the reacting group is directly involved in catalysis even though the term "essential" might imply a functional role. "Essential" residues may fall into any of several categories (Nicoli et al., 1974): (1) modification of the group(s) may affect the three-dimensional structure of the enzyme; (2) the group(s) may be in or near the active center but be nonfunctional in catalysis or binding (modification might introduce a steric or electrostatic interference with binding or catalysis); (3) the group(s) may be in the active center and be functional in catalysis or in binding.

The inactivation of luciferase by FDNB does not appear to cause any gross alterations in structure. The stoichiometry of the modification/inactivation is 1:1, and the modified and native enzymes behave identically in sedimentation velocity analysis.

A location of the reactive amino groups in or near the active center is suggested by the observations that the modification results in loss of measurable $FMNH_2$ binding and that both aldehyde binding and FMN binding protect the enzyme from modification. While it is possible that the reactive amino groups could be in a region of the enzyme other than the active

center, where modification would cause a conformational change decreasing substrate binding affinity and vice versa, on the basis of the present data, we propose that the reactive amino groups reside in or near the active center and in close spatial proximity to the reactive thiol (Nicoli et al., 1974) and the reactive imidazole (Cousineau & Meighen, 1976).

The results of our studies suggest that modification of an amino group of the β subunit is sufficient to cause inactivation of the enzyme with a loss of measurable $FMNH_2$ binding. While this observation alone does not demonstrate than an amino acid residue (residues) of the β subunit is in or near the active center, it is consistent with this interpretation. Since amino groups of both subunits appear to react rapidly with FDNB, it is likely that they occupy comparable environments. This is consistent with the observed similarity of primary sequence of the NH_2-terminal regions of the α and β subunits (Baldwin et al., 1979). In fact, the simplest explanation of these observations would be to postulate that the reactive amino groups of the two subunits exist in close proximity to each other and that modification of either one causes structural perturbations in the active center. This hypothesis would predict that, even if residues of the β subunit do not contribute directly to the active center, the active center is close to portions of the β subunit. An unambiguous proof of this hypothesis is not available, but the large body of information that has been collected regarding the residues in or near the active center of luciferase should allow application of chemical cross-linking experiments to determine the closest point of approach of the β subunit to the active center.

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