Chemical Modification of Bacterial Luciferase with Ethoxyformic Anhydride: Evidence for an Essential Histidyl Residue†

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ABSTRACT: Bacterial luciferase is a heteropolymeric protein ($\alpha\beta$) that catalyses the conversion of chemical energy to light by oxidation of a reduced flavin mononucleotide and a long chain aliphatic aldehyde. Elucidation of the specific amino acid residues involved in the enzymatic reaction is essential for understanding the mechanism of the bioluminescent reaction. Luciferase has been found to be inactivated by ethoxyformic anhydride with a second-order rate constant of 146 M^{-1} min^{-1} at pH 6.1 and 0 °C with a concomitant increase in absorbance at 240 nm due to formation of ethoxyformylhistidyl derivatives. Activity could be restored by hydroxylamine and the pH curve of inactivation indicated the involvement of a residue having a pK_a of 6.8. Both substrates, $FMNH_2$ and aldehyde, protected the enzyme against inactivation, suggesting that the modification occurred at or near the active site. Incorporation of [^{14}C]ethoxyformyl groups into luciferase indicated that inactivation resulted from the modification of about three histidyl residues, one histidine being found on the α subunit and two on the β subunit. Hybridization experiments, in which ethoxyformylluciferase, $\alpha_m\beta_m$, was complemented with native subunits, α or β, showed that the hybrid, $\alpha_m\beta$, has the same activity as $\alpha_m\beta_m$ whereas the activity of the hybrid, $\alpha\beta_m$, was close to that of the reconstituted luciferase, $\alpha\beta$. The results indicate that modification of only one histidyl residue on the α subunit inactivates luciferase and suggest that this histidyl residue plays an essential role in the mechanism of the bacterial bioluminescent reaction.

Bacterial luciferase, isolated from the bioluminescent strain, *Beneckea harveyi,* catalyzes the oxidation of a reduced flavin and a long chain aliphatic aldehyde (RCHO) to form FMN[1] and the corresponding acid, respectively, with the emission of light at 490 nm.

$$FMNH_2 + O_2 + RCHO \rightarrow FMN + RCOOH + H_2O + h\nu_{490nm}$$

In the absence of aldehyde, the enzyme catalyzes the oxidation of $FMNH_2$ with the formation of H_2O_2 (Hastings and Balny, 1975). Although it has been postulated that two reduced flavins are involved in the bioluminescent reaction (McElroy and Green, 1955; Lee, 1972; Lee and Murphy, 1975), the majority of studies support the involvement of a single reduced flavin. These studies include the kinetic determination of the number of $FMNH_2$ binding sites on luciferase (Meighen and Hastings, 1971; Watanabe and Nakamura, 1972; Watanabe et al., 1974), measurement of the binding of FMN and $FMNH_2$ to luciferase by circular dichroism or equilibrium dialysis (Baldwin, 1974a; Baldwin et al., 1975a; Becvar and Hastings, 1975), detection of 1 mol of flavin per mol of a luciferase–flavin intermediate isolated by low-temperature chromatography (Hastings et al., 1973; Hastings and Balny, 1975), and quantum yield studies (Becvar and Hastings, 1975). Early investigations clearly demonstrated the necessity for a long chain aliphatic aldehyde (Cormier and Strehler, 1953; Strehler et al., 1954); only recently has it been demonstrated that the aldehyde is oxidized to the corresponding aliphatic acid (Shimomura et al., 1972; McCapra and Hysert, 1973; Dunn et al., 1973; Vigny and Michelson, 1974). The oxidation of one molecule of aldehyde and $FMNH_2$ per catalytic cycle clearly meets the energy requirements to populate an excited state for photon emission at 490 nm as recently supported by the direct calorimetric measurement of the enthalpy of the reaction (Mangold and Langerman, 1975).

Bacterial luciferase is a heteropolymer of molecular weight 79 000, constituted of two nonidentical subunits, α and β (42 000 and 37 000, respectively; Friedland and Hastings, 1967a; Hastings et al., 1969). Although no common tryptic peptides have yet been detected (Meighen et al., 1970), a high degree of homology in the amino terminal sequence has indicated that the two subunits might have similar three-dimensional structures (Baldwin et al., 1975b). However, the role of each subunit in the bioluminescent reaction appears to be quite different. Chemical modification and hybridization experiments (Meighen et al., 1971a,b), mutant complementation (Cline and Hastings, 1972), limited proteolysis of the native enzyme (Baldwin, 1974b; Njus et al., 1974; Nicoli et al., 1976), studies on mutants with altered bioluminescent emission spectrum (Cline and Hastings, 1974), and active site studies (Nicoli et al., 1974; Nicoli and Hastings, 1974) have shown that the active site is located specifically on the α subunit. In contrast, the role of the β subunit still remains to be elucidated, although it is essential for bioluminescent activity (Friedland and Hastings, 1967a,b; Gunsalus-Miguel et al., 1972).

The investigation of the amino acid resïdues involved directly in the catalytic center of luciferase is of special interest since the bioluminescent reaction involves conversion of chemical energy to light. Chemical modification studies have demonstrated the existence of an essential sulfhydryl residue located at or near the active site on the α subunit (Nicoli et al., 1974). However, the specific role of this residue in the mechanism of bioluminescence has not yet been ascertained. Although alkylation of the sulfhydryl residue decreased or eliminated $FMNH_2$ binding to luciferase, it has now been shown that FMN can still bind to the modified enzyme with a dissociation constant not significantly different than the native enzyme

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[1] Abbreviations used: DEAE, diethylaminoethyl; EDTA, ethylenediaminetetraacetic acid; FMN, flavin mononucleotide; $FMNH_2$, reduced FMN.

(Nicoli et al., 1976). Furthermore, a luciferase mutant with a substantially altered binding constant for $FMNH_2$ still had the same reactivity toward sulfhydryl reagents, suggesting the mutation had not occurred in close enough proximity to the sulfhydryl residue to affect its reactivity (Baldwin et al., 1975c). In addition, the pH profile for flavin binding to luciferase as well as the pH dependence of the catalytic parameters showed no correlation with the ionization of the reactive sulfhydryl, leaving open the question concerning its role in the bioluminescent reaction (Nicoli et al., 1974; Nicoli and Hastings, 1974).

The study of the pH dependence of the binding affinity of reduced flavin to luciferase, however, has suggested the possible involvement of a protonated histidyl residue in flavin binding (Nicoli et al., 1974). Since a number of flavin-dependent enzymes have also been shown to have histidyl residues at the active site (Steemkamp et al., 1974; Thomé-Bau et al., 1971; Hucho et al., 1973), the effect of chemical modification of luciferase with ethoxyformic anhydride, a relatively specific histidine reagent (Melchior and Fahrney, 1970; Muhlrad et al., 1967), has been investigated. These studies have provided evidence concerning the existence of an essential histidyl residue at the active site of luciferase that may be involved in the interaction of reduced flavin with the enzyme.

Experimental Procedure

Chemicals. FMN was a gift from Sigma and its concentration was determined spectrophotometrically using a molar extinction coefficient of 12.2×10^3 M^{-1} cm^{-1} at 450 nm (Whitby, 1954). Dodecanal was obtained from Aldrich, bovine serum albumin and dithiothreitol were from Sigma, mercaptoethanol, and 1,6-hexanediamine, *N,N*-methylenebisacrylamide, and acrylamide were from Eastman. The latter reagent (acrylamide) was, however, recrystallized from chloroform (Loening, 1967) prior to use. Sodium dodecyl sulfate was purchased from British Drug House. Either ultrapure urea from Mann was used without further purification or urea from Fisher was dissolved in H_2O immediately prior to use, treated with activated charcoal, Millipore filtered, and then deionized with a mixed-bed resin (Rexyn 300, Fisher). DEAE-Sephadex A-50 was obtained from Pharmacia, Bio-Gel P-10 (50–100 mesh) from Bio-Rad, and Sepharose-4B from Sigma. All phosphate buffers were made by mixing appropriate volumes of 2.0 M K_2HPO_4 and 2.0 M NaH_2PO_4.

Ethoxyformic Anhydride. Unlabeled ethoxyformic anhydride was obtained from Eastman, whereas [1-*ethyl*-^{14}C]-ethoxyformic anhydride (0.202 Ci/mol) was synthesized by New England Nuclear according to the procedure of Melchior and Fahrney (1970). All stock ethoxyformic anhydride solutions were prepared in ethanol. The molar concentration was determined by reaction of ethoxyformic anhydride ($\simeq 10^{-4}$ M) with 0.01 M *N*-acetylhistidine in 0.1 M phosphate, pH 6.1. After 15 min of incubation at room temperature, the increase in absorbance at 240 nm was measured and the molar concentration determined based on an extinction coefficient of 3.6×10^3 M^{-1} cm^{-1} (Holbrook and Ingram, 1973).

Purification of Bacterial Luciferase. Luciferase was purified from a strain of bioluminescent bacteria, *Beneckea harveyi* (Reichelt and Baumann, 1973), previously named MAV (Hastings et al., 1969), according to the procedure of Gunsalus-Miguel et al. (1972). The enzyme was, however, purified further by anion-exchange chromatography on aminohexyl-Sepharose-4B. The anion-exchange resin was prepared by reaction of 1,6-diaminohexane with Sepharose-4B activated with cyanogen bromide (Cuatrecasas, 1970). The aminohexyl-Sepharose-4B column (1.3 × 30 cm), preequilibrated with 0.05 M phosphate, 10^{-3} M dithiothreitol, pH 7.0, was eluted with a linear gradient of 250 ml of 0.05 to 0.25 M phosphate, pH 7.0, containing 10^{-3} M dithiothreitol. The peak of luciferase activity was precipitated in 75% saturated ammonium sulfate, redissolved in a minimal volume of 0.1 M phosphate, 0.01 M dithiothreitol, pH 7.0, and stored at −20 °C. Sodium dodecyl sulfate electrophoresis (Figure 1) demonstrated only two bands corresponding to the subunits of luciferase indicating that the enzyme was greater than 95% homogeneous by this criteria. The purified luciferase had a specific activity between 1.8 and 2.5×10^{13} quanta s^{-1} mg^{-1} with dodecanal in the standard assay (see below). If decanal was used, a specific activity of 2.0×10^{14} quanta s^{-1} mg^{-1} was obtained in the standard assay. The values agree with specific activities reported for luciferase in which chemical modification of one sulfhydryl residue inactivated luciferase (Nicoli et al., 1974) and to kinetic and binding studies which showed one binding site for $FMNH_2$ (Meighen and Hastings, 1971; Becvar and Hastings, 1975). A specific activity of 3.2 to 4.3×10^{13} quanta s^{-1} mg^{-1} with dodecanal is obtained if the dithionite assay is used (Meighen and MacKenzie, 1973) in which reduced FMN is mixed with the enzyme prior to the addition of aldehyde. The protein concentration was determined spectrophotometrically on the basis of a specific absorption coefficient of 0.94 (0.1%, 1 cm) at 280 nm (Gunsalus-Miguel et al., 1972).

The subunits of luciferase (α and β) were obtained by DEAE-Sephadex chromatography of the native enzyme in 5

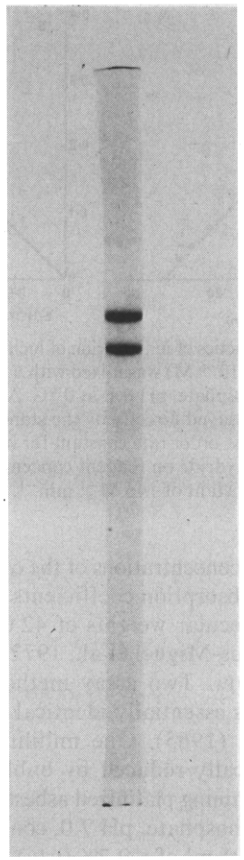

FIGURE 1: Sodium dodecyl sulfate gel electrophoresis of luciferase ($\simeq$5 μg). Electrophoresis was conducted for 5 h at 8 mA/tube and the gel stained with Coomassie blue as described in the Experimental Procedure. The upper band corresponds to the α subunit (42 000 daltons) and the lower band to the β subunit (37 000 daltons).

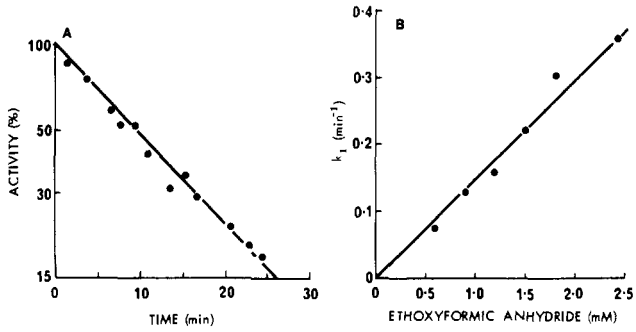

FIGURE 2: (A) The kinetics of inactivation of luciferase with ethoxyformic anhydride. Luciferase (10^{-6} M) was mixed with 6×10^{-4} M ethoxyformic anhydride in 0.1 M phosphate, pH 6.1, at 0 °C. Aliquots were withdrawn at various times and assayed directly by the standard assay. (B) Dependence of the pseudo-first-order rate constant for inactivation of luciferase with ethoxyformic anhydride on reagent concentration. The slope gives a second-order rate constant of 146 M^{-1} min^{-1}.

M urea. The molar concentrations of the α and β subunits were based on specific absorption coefficients (0.1%, 1 cm) of 1.2 and 0.65, and molecular weights of 42 000 and 37 000, respectively (Gunsalus-Miguel et al., 1972).

Luciferase Assays. Two assay methods were used. The standard assay was essentially identical with that described by Hastings et al. (1965). One milliliter of 5×10^{-5} M $FMNH_2$ (catalytically reduced by bubbling H_2 through a FMN solution containing platinized asbestos) was injected into 1.0 ml of 0.05 M phosphate, pH 7.0, containing 0.2% bovine serum albumin, 0.01 ml of a 0.2% (v/v) suspension of dodecanal in 0.1% (v/v) Triton X-100, and enzyme.

The second assay system, referred to as the dithionite assay, was identical with that described by Meighen and MacKenzie (1973) and was specifically used for samples containing hydroxylamine. In the presence of hydroxylamine, the aldehyde concentration in the assay must be increased to get maximum activity. Since high concentrations of aldehyde inhibit the standard assay but not the dithionite assay, the latter assay system was used in these cases. Light emission in both assays was measured with the photometer described by Mitchell and Hastings (1971) and was recorded graphically on a high-speed recorder (Esterline-Angus, Model S-601-S).

Ethoxyformylation of Luciferase. Luciferase was incubated at 0 °C (in ice) in 0.1 M phosphate, pH 6.1, with 10^{-3} to 10^{-4} M ethoxyformic anhydride. Aliquots were withdrawn with time and assayed directly by the standard assay. The reaction was stopped at the desired extent of inactivation by the addition of 50% by volume of 0.3 M histidine, 0.5 M phosphate, pH 7.0, 10^{-4} M dithiothreitol.

In some experiments the reaction was stopped by gel filtration of the sample on a Bio-Gel P-10 column (0.9 × 55 cm) preequilibrated with 0.1 M phosphate, pH 6.7, and eluted with the same buffer. The ethoxyformylluciferase was well resolved from any unreacted reagent and low-molecular-weight products arising from the hydrolysis of the reagent.

The specific activity and specific radioactivity of the ethoxyformylluciferase were determined either after dialysis or gel filtration. No change in specific activity was observed during either of these procedures. The number of moles of ethoxyformyl residues per mole of luciferase was based on a specific radioactivity of 0.101 Ci per mole of ethoxyformyl residues and a molecular weight of 79 000 for bacterial luciferase (Hastings et al., 1969). The relative activity of the ethoxyformylluciferase was obtained by dividing the specific activity of the modified enzyme by that for the native enzyme.

Sodium Dodecyl Sulfate Gel Electrophoresis of [^{14}C]-Ethoxyformylluciferase. Sodium dodecyl sulfate gels were prepared according to the procedure of Weber and Osborn (1969). Samples of [^{14}C]ethoxyformylluciferase in 0.5% sodium dodecyl sulfate–0.2 M mercaptoethanol were applied to each gel and electrophoresis conducted at 8 mA per tube for 6 to 7 h. The gels were fixed and stained overnight with 0.025% Coomassie brilliant blue in 25% 2-propanol–10% acetic acid. The gels were rapidly destained with 10% acetic acid–10% 2-propanol (4 to 5 h). The stained luciferase bands (α and β) were excised and then dissolved by incubation at 54 °C overnight with 6 drops of 30% H_2O_2 in sealed glass vials. After the addition of 10 ml of Aquasol to each vial the samples were counted for ^{14}C in a Packard liquid scintillation counter. The samples were routinely checked for quenching by the addition of an internal standard. Between 80 and 90% of the radioactivity applied to these gels was recovered in the α and β subunits. In some experiments, a number of gels (two to five) were sliced and the bands corresponding to each subunit pooled prior to elucidation of the amount of radioactivity in each subunit.

Results

Inactivation of Luciferase with Ethoxyformic Anhydride. The reaction of bacterial luciferase with ethoxyformic anhydride results in the loss of its enzymic activity in the bioluminescent reaction. The rate of inactivation follows first-order kinetics over a relatively wide range providing that a sufficient molar excess of reagent is added to the enzyme (Figure 2). As would be expected, the pseudo-first-order rate constant is independent of the enzyme concentration ($1–20 \times 10^{-6}$ M) but dependent on the reagent concentration giving a second-order rate constant of 146 M^{-1} min^{-1} for inactivation of luciferase. These results are consistent with a simple bimolecular reaction between ethoxyformic anhydride and luciferase resulting in inactivation.

Substrate Protection. The inactivation of luciferase by chemical modification with ethoxyformic anhydride does not directly imply that a specific residue is being modified nor that such a residue is present at the active site (Cohen, 1970). However, protection of an enzyme by substrate or substrate analogues against inactivation would suggest that the amino acid residues that are protected from modification are at or near the active site. Both substrates, $FMNH_2$ and a long chain aliphatic aldehyde, protect luciferase against inactivation with ethoxyformic anhydride. Dodecanal decreases the rate of inactivation with ethoxyformic anhydride by fivefold (Figure 3), whereas FMN and $FMNH_2$ decrease the rate of inactivation by four- and sevenfold, respectively (Figure 4). The greater protection of luciferase by $FMNH_2$ than FMN is not understood since $FMNH_2$ is chemically oxidized to FMN within a few minutes under the conditions of this experiment (Nicoli et al., 1974). However, similar results have been reported for the flavin protection of luciferase against inactivation with *N*-ethylmaleimide (Nicoli et al., 1974).

Identification of Histidine as the Modified Amino Acid. Although the reaction of ethoxyformic anhydride with proteins is relatively specific at pH 6.1 for histidyl residues (Muhlrad et al., 1967; Pradel and Kassab, 1968), other residues may also be modified in neutral or weakly alkaline media (Larrouquère, 1964; Muhlrad et al., 1967; Melchior and Fahrney, 1970; Burstein et al., 1974). Consequently, the identification of the amino acid residue(s) in luciferase, whose modification results in the loss of activity, was investigated by (a) following changes in absorbance at 240 and 280 nm of the enzyme upon modifi-

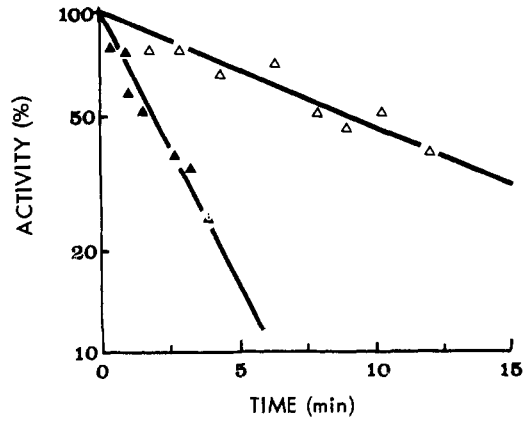

FIGURE 3: Aldehyde protection against inactivation of luciferase with ethoxyformic anhydride. Dodecanal (0.025 ml of 0.2% (v/v) in 0.1% (v/v) Triton X-100) was mixed with luciferase in 1.0 ml of 0.1 M phosphate, pH 6.1 at 0 °C; within 1 min, 10 μl of 0.3 M ethoxyformic anhydride was added (△). Aliquots were taken at various times and assayed by the standard assay. A control experiment in which no aldehyde was added is also shown (▲).

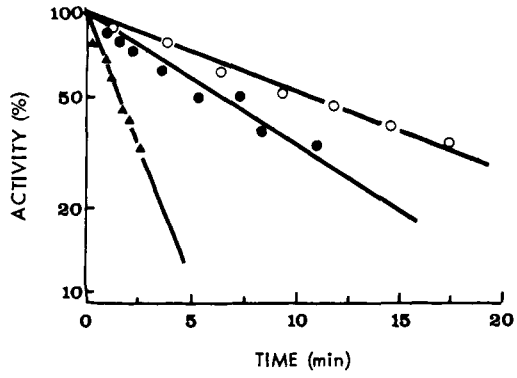

FIGURE 4: Flavin protection of luciferase against ethoxyformic anhydride inactivation. Precooled solutions (0.5 ml) of 5.2×10^{-5} M FMN (●) or 5.2×10^{-5} M $FMNH_2$ (○) at 0 °C were rapidly injected into 0.5 ml of luciferase in 0.2 M phosphate, pH 6.1, at 0 °C; within 1 min, 10 μl of 0.3 M ethoxyformic anhydride was added. An experiment in which H_2O was injected instead of flavin is also shown (▲). All experimental points were compared with controls in which no reagent had been added. The activities of the controls either remained constant or decreased less than 10% during the course of the experiments.

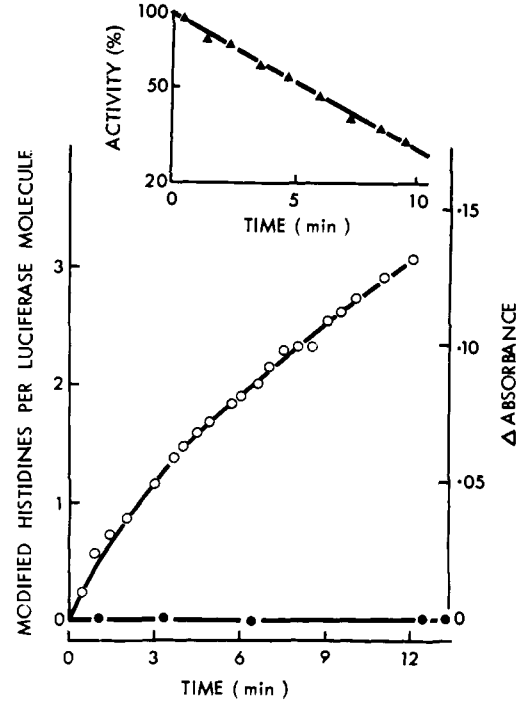

FIGURE 5: Dependence of absorbance at 240 nm (○) and 280 nm (●) on the extent of inactivation of luciferase (1.1×10^{-5} M) with ethoxyformic anhydride (9×10^{-4} M). The reaction was followed with time in a Cary 15 spectrophotometer in 0.1 M phosphate, pH 6.1, maintained at 0 °C by circulation of cold 30% glycerol around the cell compartment. The number of modified histidines (○) was based on a molar extinction coefficient of 3600 M^{-1} cm^{-1} for ethoxyformylhistidine at 240 nm (Holbrook and Ingram, 1973). At various times, aliquots were withdrawn and assayed directly for activity (insert).

TABLE I: Reactivation of Ethoxyformylluciferase by NH_2OH.[a]

Time (h)	Act. (%)
0	27
1	64
2	77
4	82
22	100

[a] Luciferase was inactivated with ethoxyformic anhydride and the reaction stopped with 27% activity remaining (see Experimental Procedure). The modified luciferase was then diluted (2:3) into a final concentration of 0.014 M mercaptoethanol, 0.1 M phosphate, 0.60 M NH_2OH, pH 7.4, and incubated at room temperature. Activity was followed with time using the dithionite assay and is given as the percentage of activity in a control of the native enzyme treated in an identical manner. The control remained constant over the first 4 h of the experiment but decreased by 50% by 22 h.

cation, (b) treatment of the inactivated enzyme with hydroxylamine, and (c) investigating the effect of pH on the rate of inactivation.

The reaction of proteins with ethoxyformic anhydride will result in an increase in absorbance at 240 nm if histidyl residues are modified (Ovadi et al., 1967) and a decrease in absorbance at 280 nm if tyrosyl residues are modified (Muhlrad et al., 1967). The dependence of the absorbance of luciferase at 240 and 280 nm upon inactivation with ethoxyformic anhydride (Figure 5) indicates that histidyl residues are modified whereas tyrosyl residues are not modified. About 1.7 histidyl residues are modified with ethoxyformic anhydride after 50% inactivation of luciferase ($t_{1/2}$ = 5.3 min).

Treatment of ethoxyformylluciferase with NH_2OH results in restoration of the original activity (Table I). Very little reactivation was observed in the absence of NH_2OH. Since NH_2OH cleaves the ethoxyformyl–histidyl bond but does not cleave the more stable ethoxyformyl–lysyl bond (Melchior and Fahrney, 1970), the inactivation of luciferase by ethoxyformic anhydride appears to be due to the modification of one or more histidyl residues. Gel filtration of the reactivated enzyme showed that between 75 and 90% of the ethoxyformyl groups (^{14}C-labeled) were released from the modified luciferase after NH_2OH treatment for 150 min, in agreement with the 80% recovery of activity in this period.

Further evidence for the modification of a histidyl residue(s) with ethoxyformic anhydride comes from the study of the rate of inactivation as a function of pH. If a histidyl residue is required for the binding of substrate or for catalysis, the rate of inactivation of the enzyme will depend on the degree of protonation of the histidine if the unprotonated histidine is mod-

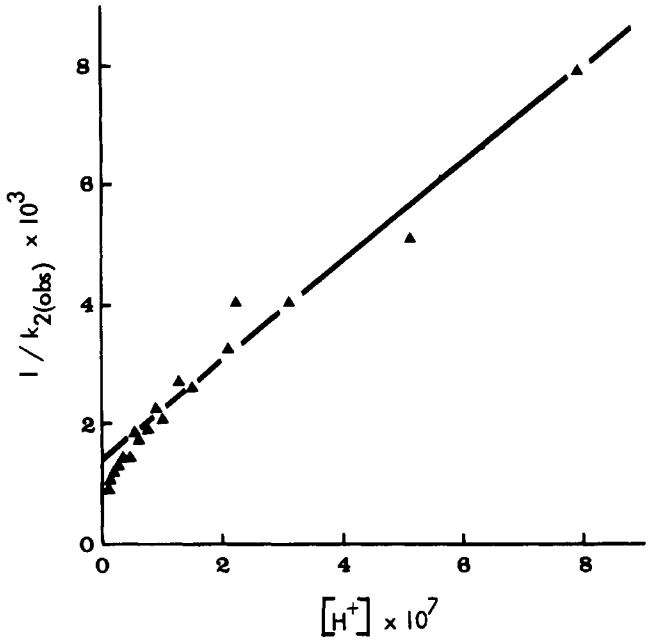

FIGURE 6: The pH dependence of inactivation of luciferase with ethoxyformic anhydride. All reactions were conducted at 0 °C in 0.037 M sodium pyrophosphate–0.5 M NaCl, adjusted to the desired pH with citric acid. The second-order rate constant, $k_{2(obsd)}$, for each pH, was obtained from the pseudo-first-order rate constant for inactivation of luciferase and the concentration of ethoxyformic anhydride and plotted as $1/k_{2(obsd)}$ vs. $[H^+]$.

ified at a substantially faster rate than the protonated histidine, as shown in the scheme below:

$$\text{E-HIS-H}^+ \overset{K_a}{\rightleftarrows} \underset{+\ H^+}{\text{E-HIS}} \xrightarrow[X]{k_2} \text{E-HIS-X} \qquad (1)$$

The observed second-order rate constant for inactivation ($k_{2(obsd)}$) will thus be given by the equation below:

$$k_{2(obsd)} = \frac{k_2 K_a}{K_a + [H^+]} \qquad (2)$$

where k_2 is the second-order rate constant for modification of the unprotonated histidine and K_a is the apparent dissociation constant of the acidic form of histidine. Equation 2 can be rewritten as:

$$\frac{1}{k_{2(obsd)}} = \frac{1}{k_2} + \frac{[H^+]}{k_2 K_a} \qquad (3)$$

A plot of $1/k_{2(obsd)}$ vs. $[H^+]$ should thus give a straight line with an intercept of $1/k_2$ and slope equal to $1/(k_2 K_a)$ (Figure 6). From the intercept and slope, k_2 was calculated to be 720 M^{-1} min^{-1} and the apparent pK_a of the histidyl residue(s) was found to be 6.8. Similar values for the pK_a of histidyl residues have been obtained by modification of histidyl residues in other enzymes (Dickenson and Dickinson, 1975; Holbrook and Ingram, 1973). It should be pointed out that the straight line was only drawn through the points at neutral or acidic pH (<7.3) since curvature downwards can clearly be observed at alkaline pH (pH >7.5) reflecting the modification of other essential amino acids in luciferase. The modification of amino acid residues other than histidine above pH 7 with ethoxyformic anhydride has also been observed in other enzymes (Larrouquère, 1964; Holbrook and Ingram, 1973; Muhlrad et al., 1967; Ovadi and Keleti, 1969; Elodi, 1972) and emphasizes the importance of carrying out the modification of histidine in enzymes at pH values below 7.

TABLE II: Stability of Ethoxyformylluciferase.[a]

Time (h)	Radioact. (%)	Act. (%)
0	100	32
4	6	26
7	2	29
22	0.91	29
26	0.98	27
30	0.93	28

[a] Luciferase was inactivated with [^{14}C]ethoxyformic anhydride (0 °C, pH 6.1) and the reaction stopped with 32% activity remaining (see Experimental Procedure). The reaction mixture was then dialyzed against 0.05 M phosphate–10^{-4} M dithiothreitol, pH 7.0. The percentage of radioactivity remaining in the dialysis bag and the activity of the ethoxyformylluciferase were measured with time.

Stoichiometry of the Reaction. The number of moles of reagent required for inactivation of luciferase was elucidated by measuring the amount of ethoxyformic anhydride incorporated into the protein as a function of the extent of inactivation. In this experiment, luciferase was inactivated to different extents with [^{14}C]ethoxyformic anhydride and the radioactivity not incorporated into the protein removed by gel filtration or dialysis (Table II). Dialysis of the reaction mixture for periods of 20 h or longer removed no further radioactivity and had little effect on activity (Table II) showing that the ethoxyformyl–histidyl bonds were relatively stable under these conditions (4 °C, pH 7.0). The percentage of enzyme activity remaining was then plotted as a function of the moles of radioactive label incorporated into the enzyme (Figure 7). Assuming that a linear extrapolation can be made to zero activity using only the data for low degrees of modification, the results indicate that it is only necessary to modify three or less histidyl residues with ethoxyformic anhydride to inactivate luciferase. The number of modified histidines based on the change in absorbance at 240 nm exactly follows the moles of radioactive label introduced into luciferase (Figure 7). This result strongly suggests that only histidyl residues are being modified in luciferase during the course of inactivation with ethoxyformic anhydride.

Since bacterial luciferase contains two nonidentical subunits, α and β (Hastings et al., 1969; Gunsalus-Miguel et al., 1972), which can be separated in the presence of denaturants, the moles of reagent incorporated into each subunit can therefore be determined. DEAE-Sephadex chromatography of ethoxyformylluciferase (2.1 ethoxyformyl groups per molecule of luciferase) in 5 M urea resulted in the elution of three peaks of radioactivity (Figure 8). The two major peaks, containing 60 and 30% of the applied radioactivity, correspond to the absorbance peaks for the β and α subunits of luciferase, respectively. The small initial peak of radioactivity, containing 10% of the appplied radioactivity, is believed to arise from hydrolysis of ethoxyformyl–histidyl bonds during the course of the experiment. This conclusion is supported by its position of elution in the dead volume, the absence of absorbance at this position, the capability of this material to pass through dialysis membranes, and the known lability of the ethoxyformyl–histidyl bond (Melchior and Fahrney, 1970). If it is assumed that no preferential loss of label had occurred from either subunit, then 33% of the ethoxyformyl residues originally incorporated into luciferase must have been present on the α subunit and 67% on the β subunit.

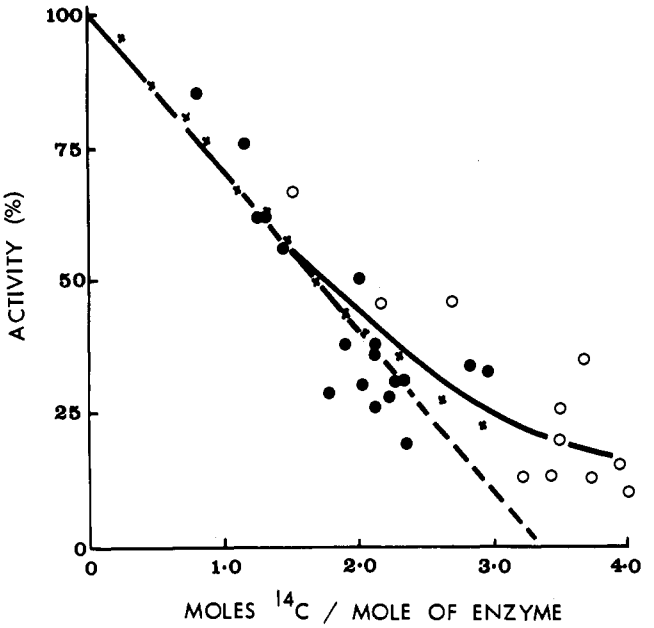

FIGURE 7: The percentage of activity remaining as a function of moles of [^{14}C]ethoxyformic anhydride incorporated into luciferase. Luciferase ($1-2 \times 10^{-5}$ M) and ethoxyformic anhydride ($3-6 \times 10^{-4}$ M) were mixed in 0.1 M phosphate, pH 6.1, at 0 °C, the reaction was stopped at different times, and excess reagent was removed by dialysis (●) or gel filtration (○) (see Experimental Procedure). A straight line with the minimum standard deviation has been drawn through the points at low degrees of modification (<2.5 mol incorporated per mol of enzyme). The number of histidyl residues modified per molecule of luciferase based on the change in absorbance at 240 nm (see Figure 5) has also been plotted as a function of inactivation of luciferase (X).

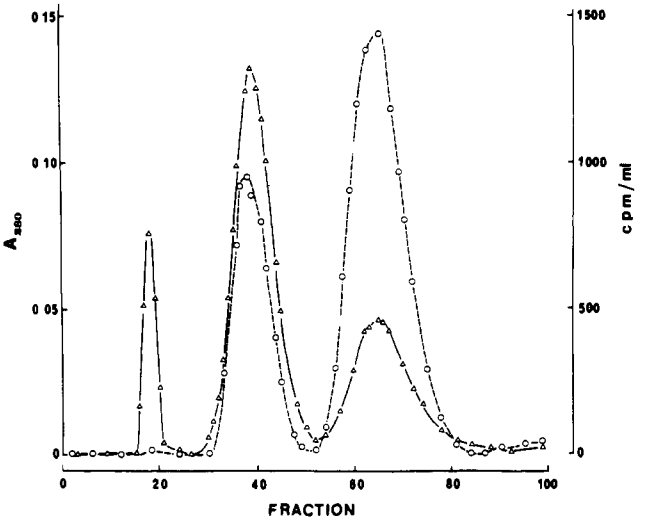

FIGURE 8: Separation of the α and β subunits in ethoxyformylluciferase by DEAE-Sephadex chromatography in urea. Luciferase was inactivated with [^{14}C]ethoxyformic anhydride (0 °C, pH 6.1) to about 40% activity, the reaction stopped, and the modified luciferase precipitated by dialysis at 4 °C against 75% saturated ammonium sulfate, 0.1 M phosphate, pH 7.0. The precipitate was collected by centrifugation and the pellet redissolved by dialysis against 0.05 M phosphate-10^{-4} M dithiothreitol, pH 7.0. Luciferase was then dissociated by the addition of urea to give a final solution of enzyme in 5 M urea, 10^{-3} M dithiothreitol, 10^{-3} M EDTA, 0.05 M phosphate, pH 7.0. The sample was applied to a 0.9 × 45 cm DEAE-Sephadex A-50 column, preequilibrated with 5 M urea, 10^{-3} M dithiothreitol, 10^{-3} M EDTA, 0.05 M phosphate, pH 7.0, and eluted at 4 °C with a 145-ml linear gradient of 0.05 to 0.12 M phosphate, pH 7.0, containing 5 M urea, 10^{-3} M dithiothreitol, 10^{-3} M EDTA. Fractions of 1.4 ml were collected and the radioactivity (△) and absorbance at 280 nm (○) measured across the column.

This conclusion was also reached for luciferase molecules modified to different extents with ethoxyformic anhydride by separation of the modified α and β polypeptides by sodium dodecyl sulfate gel electrophoresis and measurement of the distribution of radioactivity in the two subunits. Although this approach is not as accurate as DEAE-Sephadex chromatography in urea due to the lower amount of material that can be analyzed, it does provide a more rapid method for determining the distribution of radioactivity in a large number of samples. The results in Figure 9 show that, within the range of 0.5 to 3.7 ethoxyformyl residues per molecule of luciferase, 40% of the recovered radioactive label is present in the α subunit and 60% in the β subunit. As the lability of the ethoxyformyl-histidyl bonds should be similar in the unfolded α and β polypeptide chains and 80 to 90% of the applied radioactivity was recovered in the two subunits in these experiments, it can be concluded, without introduction of a large error, that the distribution of radioactivity between the subunits is essentially the same before and after electrophoresis.

Since previous experiments (see Figure 7) indicated that less than 3.3 histidyl residues modified by ethoxyformic anhydride are involved in the inactivation of luciferase, 1.1 to 1.3 histidyl residues (33 to 40% of 3.3 residues) should be modified on the α subunit and 2.0 to 2.2 histidyl residues modified on the β subunit. Consequently, it would appear that approximately three histidyl residues (two on β, one on α) in luciferase are modified at similar rates with ethoxyformic anhydride and that modification of at least one of these residues results in inactivation.

Complementation Studies. The determination of whether the histidyl residues on the α or β subunit are essential for activity can be deduced by complementation of the inactive

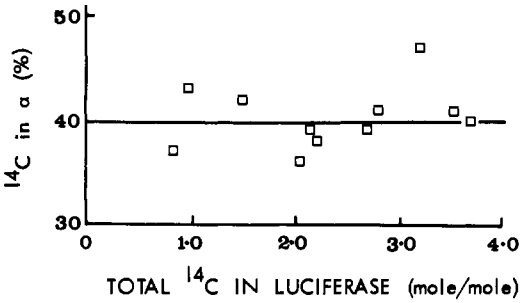

FIGURE 9: Distribution of radioactivity in the two subunits of luciferase as a function of the extent of modification with [^{14}C]ethoxyformic anhydride. Luciferase was reacted with [^{14}C]ethoxyformic anhydride to different extents, the reaction stopped, the sample dialyzed against 0.05 M sodium phosphate, pH 7.0, and the number of moles of reagent covalently incorporated into luciferase was determined. Sodium dodecyl sulfate was added to give a final concentration of 0.5%, and the subunits were separated by gel electrophoresis in sodium dodecyl sulfate as described in the Experimental Procedure. The data are given as the percentage of the total recovered radioactivity in the α subunit.

modified luciferase with native subunits. If one of the native subunits (α or β) is mixed in urea with the modified luciferase ($\alpha_m\beta_m$) and the urea removed, then the corresponding hybrid, $\alpha\beta_m$, can be produced (e.g., $\alpha_m\beta_m + \alpha \rightarrow \alpha\beta_m$). After correction of the resulting activity for recombination of α_m and β_m (i.e., 25% of β_m in $\alpha_m\beta_m$ will complement with α_m if $\alpha/\alpha_m = 3$), the specific activity of the hybrid was then calculated based on the maximum number of moles of $\alpha\beta_m$ or $\alpha_m\beta$ that could be formed (moles of α_m or β_m added). Complementation of $\alpha_m\beta_m$ with an excess of β subunit gives a specific activity for the hybrid, $\alpha_m\beta$, of only 80 LU/mg (Figure 10A), which is

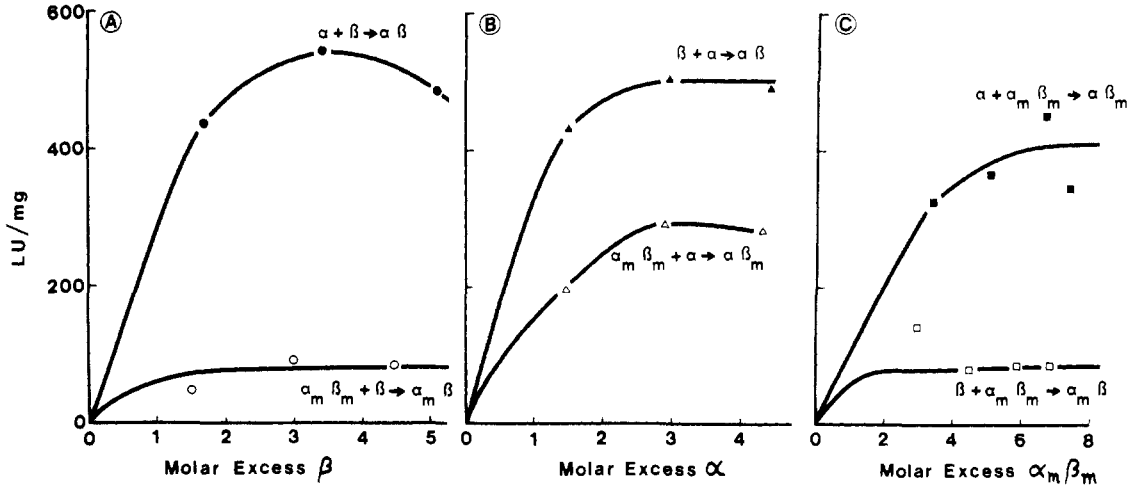

FIGURE 10: Complementation of ethoxyformylluciferase, $\alpha_m\beta_m$ (10% activity; 4.2 mol of [^{14}C]ethoxyformic anhydride incorporated). A fixed amount of $\alpha_m\beta_m$ (2.2×10^{-10} mol in 70 μl of 5 M urea) and varying amounts of α ($3.2\text{–}9.6 \times 10^{-10}$ mol in 0.1 to 0.3 ml of 5 M urea) or β subunit ($3.2\text{–}9.6 \times 10^{-10}$ mol in 0.06 to 0.18 ml of 5 M urea) were mixed in 1.0 ml of 0.1 M phosphate, 0.001 M EDTA, 0.01 M dithiothreitol, 0.2% bovine serum albumin, pH 7.0, and reconstituted by dialysis for 4 h at 6 °C against 0.1 M phosphate, 0.01 M EDTA, 0.014 M mercaptoethanol, pH 7.0. The activity was measured and plotted as a function of the molar excess of β (A; ○) or α (B; △) over $\alpha_m\beta_m$. The specific activity was calculated based on the moles of $\alpha_m\beta_m$ added. No significant activity was contributed by the addition of the α or β subunits. The control experiments in which a fixed amount of α subunit was titrated with an excess of β subunit (A; ●) and vice versa (B; ▲) give a maximum specific activity of 525 LU/mg for reconstituted luciferase. The specific activities were determined from the amount of luciferase that could be formed based on the amount of the subunit present in limiting quantities. In the reverse experiments (C), a constant amount of α (1.9×10^{-10} mol in 60 μl of 5 M urea; ■) or β subunit (2.2×10^{-10} mol in 40 μl of 5 M urea; □) and varying amounts of $\alpha_m\beta_m$ ($6.3\text{–}14.5 \times 10^{-10}$ mol in 0.2 to 0.46 ml of 5 M urea) were reconstituted as described above. The experimental data were corrected for background activity of reconstituted $\alpha_m\beta_m$.

identical with the specific activity for reconstituted $\alpha_m\beta_m$ (76 LU/mg). In contrast, complementation of $\alpha_m\beta_m$ with an excess of α subunit gives a specific activity for the hybrid, $\alpha\beta_m$, of 290 LU/mg (Figure 10B). The calculated specific activities for the hybrids are clearly minimum values since all of the modified subunit would not be complemented with the corresponding native subunit at the relatively low molar excess of native to modified subunits used in the present experiments. However, higher molar excess of native subunits could not be used since a lower recovery of activity resulted.

The complementation experiments can also be conducted in the reversed manner by titration of a fixed amount of the native subunit (α or β) with a molar excess of $\alpha_m\beta_m$. The specific activity, in this case, is based on the moles of α or β subunit originally added and is also corrected for background activity resulting from complementation of α_m and β_m to form $\alpha_m\beta_m$. Complementation of the β subunit with an excess of $\alpha_m\beta_m$ gives a specific activity for $\alpha_m\beta$ of only 80 LU/mg in agreement with the previous results in Figure 10A, whereas the complementation of the α subunit with an excess of $\alpha_m\beta_m$ gives a specific activity for $\alpha\beta_m$ of 390 LU/mg (Figure 10C). The control experiments in which a fixed amount of β subunit is titrated with excess α subunit (Figure 10A) or vice versa (Figure 10B) both give a specific activity for reconstituted native luciferase ($\alpha\beta$) of 525 LU/mg. Both sets of complementation experiments indicate that a luciferase molecule containing ethoxyformyl-histidyl residues only in the α subunit ($\alpha_m\beta$) has essentially the same specific activity (80 LU/mg) as $\alpha_m\beta_m$ (76 LU/mg), whereas a luciferase molecule with ethoxyformyl-histidyl residues on the β subunit ($\alpha\beta_m$) has a specific activity (290 to 390 LU/mg) closer to that of the native enzyme (525 LU/mg). It should be noted, however, that the specific activities for the hybrids are minimum values since it was assumed in the calculation of these specific activities that the maximum amount of hybrid was formed, whereas it is clear that complementation must be incomplete (e.g., only 75% of β_m in $\alpha_m\beta_m$ will be complemented with α at $\alpha/\alpha_m = 3$). If the maximum specific activities observed experimentally for the hybrids are corrected for incomplete complementation, then values of 110 LU/mg for $\alpha_m\beta$ and 400 to 500 LU/mg for $\alpha\beta_m$ would be obtained. Consequently, it would appear that modification of the histidyl residues on the α subunit rather than the β subunit results in inactivation of luciferase. Since the results given previously indicate that a maximum of 1.3 histidyl residues on the α subunit modified with ethoxyformic anhydride are involved in the inactivation of luciferase, the results provide evidence for a single essential histidyl residue located on the α subunit.

Discussion

The reaction of proteins with most chemical reagents can generally lead to the modification of a wide variety of amino acid side chains. Although ethoxyformic anhydride can react with the side chains of histidine, lysine, tyrosine, cysteine, serine, and arginine as well as the α-amino group of amino acids (Melchior and Fahrney, 1970; Muhlrad et al., 1967), the side chain of histidine can be specifically modified at a pH near 6 with a relatively low ethoxyformic anhydride concentration. This conclusion is based on a wide body of evidence that has accumulated over the last 10 years showing that tyrosine side chains may react at pH 7 but not at lower pH values (Muhlrad et al., 1967; Burstein et al., 1974); cysteinyl residues have not been modified at pH 6 in a variety of proteins (Pradel and Kassab, 1968; Dann and Britton, 1974; Grillo, 1971; Tudball et al., 1972; Huc et al., 1971; Miles and Kumagai, 1974; Kumagai et al., 1975), although modification of model sulfhydryl compounds has been observed (Larrouquère, 1965; Muhlrad et al., 1967); arginine modification has only been reported in model compounds (Muhlrad et al., 1967); serine modification appears to occur only at the active site serine of chymotrypsin to form a highly unstable derivative (Melchior and Fahrney, 1970); and amino groups will generally not react at low pH values (Muhlrad et al., 1967; Dann and Britton, 1974). Although amino groups can, in some cases, be modified with

ethoxyformic anhydride at pH values below 7 (Melchior and Fahrney, 1970; Wells, 1973; Burstein et al., 1974), the second-order rate constant at pH 6.0 and 25 °C for modification of a highly reactive lysyl residue in phospholipase A_2 (Wells, 1973) is relatively low (15 M^{-1} min^{-1}) compared with the rate of modification of the imidazole ring in model compounds ($\simeq$150 to 200 M^{-1} min^{-1}) at this pH and temperature (Holbrook and Ingram, 1973; Elodi, 1972; Dickenson and Dickinson, 1975). In addition, the inactivation of proteins by modification of histidyl residues with ethoxyformic anhydride has generally resulted in a second-order rate constant for inactivation greater than 10 M^{-1} min^{-1} at pH 6, ranging from about 10 M^{-1} min^{-1} at pH 6.2, 25 °C, for the inactivation of thermolysin (Burstein et al., 1974) to 1800 M^{-1} min^{-1} at pH 6 and 20 to 25 °C for the modification of the hyperreactive histidyl residues in pig heart lactate dehydrogenase (Holbrook and Ingram, 1973) and yeast alcohol dehydrogenase (Dickenson and Dickinson, 1975). If the rate constant for inactivation with ethoxyformic anhydride at pH 6 is sufficiently high, it may be reasonable to conclude that modification of a histidyl residue is responsible.

In the present experiments, luciferase was inactivated in 0.1 M phosphate, pH 6.1, at 0 °C with ethoxyformic anhydride. The specificity of this reaction for histidyl residues was supported by (a) comparison of the absorbance of the native and modified enzyme at 240 and 280 nm, (b) the reversal of inactivation by NH_2OH, (c) the dependence of the reaction rate on a residue with a pK_a equal to 6.8, in agreement with the pK values reported for histidyl residues modified with ethoxyformic anhydride in other proteins (Holbrook and Ingram 1973; Dickenson and Dickinson, 1975), (d) the direct relationship between moles of ^{14}C incorporated and the moles of histidine modified based on changes in the absorbance at 240 nm, and (e) a relatively high second-order rate constant for inactivation of 146 M^{-1} min^{-1} at 0 °C and pH 6.1, a rate that is as large as that observed for model imidazole compounds at higher temperatures (25 °C) and this pH. In addition, prior modification of the sulfhydryl residues had no effect on the modification of luciferase with ethoxyformic anhydride (Cousineau and Meighen, manuscript in preparation). These results would indicate that only histidyl residues were modified in the present experiments and this modification resulted in inactivation.

An advantage of conducting the reaction for short times at pH 6.1 and 0 °C in phosphate buffer is that a significant percentage of the reagent is not hydrolyzed during the course of the reaction and consequently first-order kinetics are observed for inactivation of luciferase if a sufficient molar excess of reagent is added. Although the half times for decomposition of ethoxyformic anhydride at pH 7 and 20 to 25 °C are reported to be between 8 and 25 min (Berger, 1975; Larrouquère, 1964; Melchior and Fahrney, 1970), the rate of breakdown of ethoxyformic anhydride in aqueous media is strongly dependent on buffer composition, pH, and temperature (Berger, 1975). Both at lower pH and lower temperature the rate of decomposition is significantly decreased (Berger, 1975).

The proposal that inactivation of luciferase arises from modification of a single histidyl residue on the α subunit was based on measurement of the moles of reagent incorporated into each subunit (α or β) and the regeneration of activity by complementation of the modified enzyme with the α subunit but not the β subunit. Since the results indicate that modification of a single histidyl residue on the α subunit results in inactivation, and both substrates, $FMNH_2$ and aldehyde, protect against this inactivation, it can be suggested that the inactivation of luciferase with ethoxyformic anhydride is due to the modification of an essential histidyl residue located at the active site of luciferase on the α subunit. The results are consistent with a positively charged histidine being involved in the binding of reduced flavin at the active site (Nicoli et al., 1974).

However, an alternative possibility cannot yet be eliminated, in particular, that the essential histidyl residue is not directly at the active site. If flavin binding, as recently proposed by Nicoli et al. (1976), results in a conformational change in luciferase, then it is possible that the rate of inactivation of luciferase by ethoxyformic anhydride could be decreased even if an essential histidyl residue was not directly at the active site. Chemical modification of this essential histidyl residue would then cause inactivation either by interfering with the conformational change induced by substrate, thus decreasing the binding affinity of the substrates or preventing the correct alignment of the catalytic residues, or possibly by causing a conformational change itself. Only by further investigation of the active site and the mechanism of the reaction can such possibilities be eliminated or perhaps proven.

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