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Bacterial Luciferase: Demonstration of a Catalytically Competent Altered Conformational State following a Single Turnover†

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ABSTRACT: Ziegler-Nicoli et al. [Ziegler-Nicoli, M., Meighen, E. A., & Hastings, J. W. (1974) *J. Biol. Chem. 249*, 2385-2392] reported that a highly reactive cysteinyl residue on the α subunit of bacterial luciferase resides in or near the flavin binding site such that the enzyme–flavin complex is protected from inactivation by alkylating reagents. These authors also observed that injection of reduced flavin mononucleotide ($FMNH_2$) into an air-equilibrated solution of enzyme protected the enzyme from alkylation for much longer than the lifetime of the 4a-peroxydihydroflavin intermediate resulting from reaction of enzyme-bound $FMNH_2$ with O_2. Two related explanations were offered: either (1) the product flavin mononucleotide dissociated from the enzyme much more slowly following a catalytic cycle than would be predicted from the K_d measured by equilibrium binding or (2) the enzyme itself, without bound flavin, was in an altered conformational state in which the thiol was less reactive following a catalytic cycle. Either explanation involves a slow return of the enzyme to its initial state following a catalytic cycle. We have investigated this phenomenon in more detail and found that rapid removal of the flavin from the enzyme by chromatography following catalytic turnover did not return the enzyme to its original state of susceptibility to either alkylating reagents or proteolytic enzymes. The flavin-free enzyme returned to the susceptible conformation with a half-time of ca. 25 min at 0 °C. Inactivation of the enzyme at intermediate times of relaxation by either a proteolytic enzyme or an alkylating reagent showed biphasic kinetics, indicative of a mixture of the protected and susceptible forms. Our results demonstrate that the enzyme is in an altered conformational state following a single catalytic cycle and that this altered conformational state slowly relaxes to a conformer resembling the original in susceptibility to alkylating reagents and proteolytic enzymes.

Bacterial luciferase is a flavin monooxygenase which catalyzes the oxidation of reduced flavin mononucleotide ($FMNH_2$) and long-chain aldehydes in the presence of oxygen, producing blue-green light (λ_{max} 490 nm) [see Ziegler & Baldwin (1981) for a review]. The structure of the enzyme has been studied in some detail, primarily through the use of chemical modification and mutant enzyme analyses. Ziegler-Nicoli et al. (1974) have shown that the enzyme is rapidly inactivated by alkylating reagents due to the high reactivity of a specific thiol in or near the active center. Modification of this thiol, which resides on the α subunit, results in loss of measurable reduced flavin binding, while binding of oxidized flavin protects the thiol from the alkylating reagents. Another protection experiment reported by these authors led to an unexpected result. Injection of $FMNH_2$ into a solution of luciferase in air-equilibrated buffer resulted in the formation of a species of enzyme that was inactivated with *N*-ethylmaleimide in a pseudo-first-order reaction with a half-time of 40 min under conditions in which the native enzyme alone

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would be inactivated with a half-time of <4 min. Enzyme in the presence of the same concentration of flavin mononucleotide (FMN) was inactivated with a half-time of 15 min. The product of the reaction of $FMNH_2$ with O_2 on the enzyme, the 4a-peroxydihydroflavin (intermediate II), had a half-life of 4 min under the conditions of their experiment, only one-tenth the time of the reported protection. These authors suggested that the product of FMN from the reaction might be bound differently from FMN bound from solution and dissociate only slowly from the enzyme.

Mangold & Langerman (1975) have studied the bioluminescence reaction with the luciferase from *Vibrio harveyi* using flow microcalorimetry. The conditions of their experiments were similar to those of Ziegler-Nicoli et al. (1974). Mangold and Langerman found that reaction of $FMNH_2$ with O_2 in the presence of luciferase resulted in a rapid phase of heat release, consistent with the decay of the intermediate II, followed by a slow latent heat with a half-life of 40 min, similar to the long-lived protection observed by Ziegler-Nicoli et al. The same long-lived protection has been reported by Cousineau & Meighen (1976), who were studying the inactivation of the luciferase by the histidinyl-directed reagent ethoxyformic anhydride, but was not observed when the enzyme was being inactivated by 2,4-dinitrofluorobenzene, which derivatizes the α-amino groups (Welches & Baldwin, 1981).

Becvar et al. (1978), who were studying the decay of intermediate II by spectroscopic techniques, reported slow secondary spectral changes with the *Vibrio* (*Photobacterium*) *fischeri* enzyme reminiscent of the continued protection of the reactive thiol against alkylation long after the decay of intermediate II reported by Ziegler-Nicoli et al. (1974) for the enzyme from *Vibrio* (*Beneckea*) *harveyi*. Becvar et al. observed no comparable slow secondary spectral changes with the *V. harveyi* enzyme but suggested that the *V. harveyi* enzyme had a 10-fold higher binding affinity for FMN following decay of intermediate II than did the original enzyme.

The purpose of this paper is to report our investigations of the apparent long-term protection of the luciferase from *V. harveyi* from alkylation following decay of intermediate II. The approach that we took to determine whether a structural change might have occurred was to investigate the accessibility to proteases of peptide bonds within a region of the α subunit thought to comprise residues in close proximity both to the reactive thiol and to the active center (Baldwin et al., 1978). Our experiments show that the enzyme, when chromatographically resolved from the flavin, still retains the protected character not only from alkylating reagents but also from proteolytic enzymes as well, suggesting that the enzyme following turnover is in a structurally altered ("protected") state which relaxes slowly to the original conformation.

Materials and Methods

Chemicals. Phosphate buffers were prepared by mixing appropriate volumes of 2 M stock solutions of K_2HPO_4 and NaH_2PO_4. *N*-Ethylmaleimide, FMN, *n*-decanal, Sephadex G-25, and phenylmethanesulfonyl fluoride were obtained from Sigma Chemical Co., α-chymotrypsin was from Worthington Chemical Co., and sodium dithionite was from Fisher Scientific.

Purification and Assay of Luciferase. Cells of *Vibrio harveyi* (strain B392, wild type) were grown and the luciferase was purified as previously described by Hastings et al. (1978). Luciferase activity was measured by the standard $FMNH_2$ injection assay (Hastings et al., 1978), and the enzyme concentration was determined by using an absorption coefficient of 0.94 mL mg^{-1} at 280 nm and a molecular weight of 79 000 (Gunsalus-Miguel et al., 1972). Light emission was measured as a function of maximum light intensity (I_0) as monitored by a photomultiplier photometer (Mitchell & Hastings, 1971).

"Protected" luciferase species were prepared by a modification of the procedure described by Kurfurst et al. (1984). In this procedure, luciferase (200 μL, 0.11 mM) was mixed with FMN (25 μL, 4.8 mM) and the solution reduced with excess sodium dithionite. To start the bioluminescent reaction, the mixture was applied to an aldehyde-equilibrated (0.01% v/v sonicated decanal) Sephadex G-25 column (1 × 25 cm) in 350 mM phosphate buffer, pH 7.0 at 0 °C. As soon as the sodium dithionite was separated from the reaction mixture, a bright band of luminescence could be seen, indicating that the bioluminescent reaction had been initiated. The light emission persisted after elution of the protein fraction (1.0 mL), which allowed easy collection and transfer to a cuvette for measurement of either fluorescence or absorption spectra. The elution time of excess FMN and sodium dithionite indicated ample separation from the protein fraction. Absorbance measurements were made with a Kontron Model 810 spectrophotometer, and fluorescence measurements were taken with an SLM Model 8000 spectrofluorometer. Both instruments were fitted with circulating water baths for temperature regulation.

Flavin Protection Studies. FMN concentrations were determined spectrophotometrically by using an extinction coefficient of 12 200 M^{-1} cm^{-1} at 450 nm (Whitby, 1953). Experiments involving flavin protection of luciferase against inactivation with *N*-ethylmaleimide were done as described by Ziegler-Nicoli et al. (1974). A slight modification was that all flavin species were injected into the sample as $FMNH_2$, and for oxidized flavin protection, the injected $FMNH_2$ was allowed to oxidize before addition of luciferase. Concentrations of reagents and luciferase are given in the figure legends. Flavin protection against proteolytic digestion with α-chymotrypsin (40 μL of 5 mg/mL in 1.0-mL reaction volume) was done in the same manner. When luciferase activity had reached ~1%, 10 μL of phenylmethanesulfonyl fluoride (7 mg/mL in 2-propanol) was added to inhibit α-chymotrypsin activity. A sample was then analyzed by sodium dodecyl sulfate ($NaDodSO_4$) gel electrophoresis to determine the size of the resulting fragments (Laemmli, 1970).

Results

Our primary hypothesis, based on the intermediate II thiol protection experiments of Ziegler-Nicoli et al. (1974), was that following a cycle of $FMNH_2$ binding, O_2 reaction, and intermediate II decay, the enzyme must reside in an altered conformational state, regardless of whether the product FMN were released slowly or not. Following catalytic turnover, the enzyme must return ultimately to its original state, since the enzyme functions catalytically within the cell. Our hypothesis suggested a specific outcome to a slight alteration of the experiment of Ziegler-Nicoli et al. If the enzyme following decay of intermediate II were to exist in an altered conformational state that relaxed slowly to the original conformation, both conformations should be evident at intermediate times. To determine the existence of both susceptible and protected forms at intermediate times, $FMNH_2$ was injected into an aerobic solution of luciferase (both at 0 °C), and at either 30 s (as in the experiment of Ziegler-Nicoli et al.) or 20 min, *N*-ethylmaleimide was added to the luciferase and the time course of inactivation of the luciferase monitored as described (Ziegler-Nicoli et al., 1974) (Figure 1; note that the time axis refers to the time elapsed following mixing of enzyme with

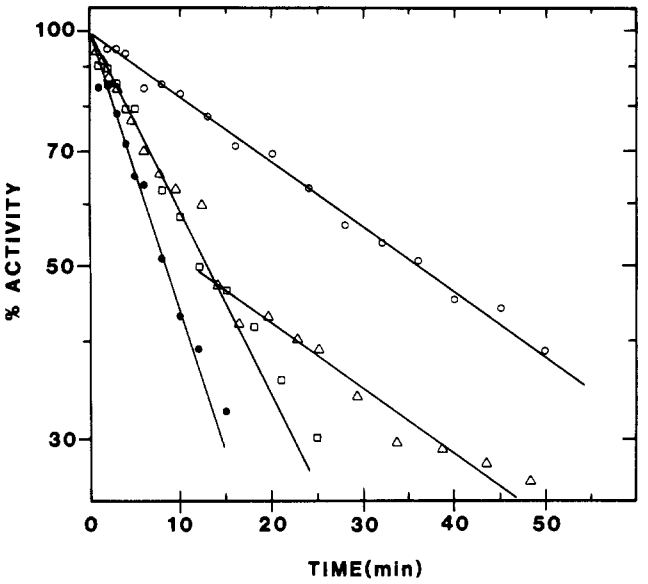

FIGURE 1: Inactivation of native and protected luciferase by *N*-ethylmaleimide. Protected enzyme was generated by injection of 0.5 mL of 48 μM $FMNH_2$ into 0.49 mL of air-equilibrated luciferase. All solutions were in 350 mM phosphate, pH 7.0, and at 0 °C. The time axis refers to elapsed time following addition of *N*-ethylmaleimide to the particular enzyme solution. The final solution concentrations were 1.7 μM luciferase, 1.0 mM *N*-ethylmaleimide and 24 μM flavin. *N*-Ethylmaleimide was added either 30 s (○) or 20 min (△) after flavin injection. For the control experiment showing protection by FMN and H_2O_2 (□), the $FMNH_2$ as injected into air-equilibrated buffer containing *N*-ethylmaleimide without enzyme and the reaction started by addition of enzyme at zero time. The second control reaction had buffer injected instead of $FMNH_2$, followed by addition of *N*-ethylmaleimide at time 0 (●). First-order rate constants were 0.083 min^{-1} without flavin, 0.053 min^{-1} in the presence of FMN and H_2O_2 [i.e., the control (□) and the fast phase of the reaction in which *N*-ethylmaleimide was added 20 min after $FMNH_2$], and 0.019 min^{-1} for the protected enzyme [i.e., the experiment in which *N*-ethylmaleimide was added 30 s after $FMNH_2$ (○) and the slow phase of the experiment in which *N*-ethylmaleimide was added 20 min after $FMNH_2$ (△)].

N-ethylmaleimide). When the reaction mixture, consisting initially of luciferase and $FMNH_2$ in air-equilibrated buffer, was allowed to incubate for 20 min at 0 °C prior to addition of *N*-ethylmaleimide, the resulting inactivation was biphasic, demonstrating the existence of at least two enzymatic forms. The initial phase followed the same rate as the control reaction mixture containing FMN and H_2O_2 resulting from nonenzymic oxidation of the reduced flavin, while the second phase followed closely the rate of inactivation of the protected form of the enzyme seen in the reaction mixture with immediate (30 s) addition of *N*-ethylmaleimide. The second phase became apparent about 15 min after addition of the *N*-ethylmaleimide, or about 35 min after injection of $FMNH_2$ into the luciferase. The protected enzyme (from the immediate, i.e., 30 s, addition of alkylating reagent) had decayed to about 50% activity in 35 min, in good agreement with the ca. 48% activity remaining when the second phase of inactivation of the biphasic reaction (from the addition of alkylating reagent after 20 min) became apparent.

Earlier experiments have suggested that the reactive thiol resides near the protease-labile region of the α subunit (Rausch et al., 1982) and that alterations in protease sensitivity might be an excellent means of detecting conformational changes in the vicinity of the active center (Baldwin et al., 1976). We therefore used sensitivity of the enzyme to α-chymotrypsin following decay of intermediate II to determine the existence of an altered conformational state (Figure 2). This experiment

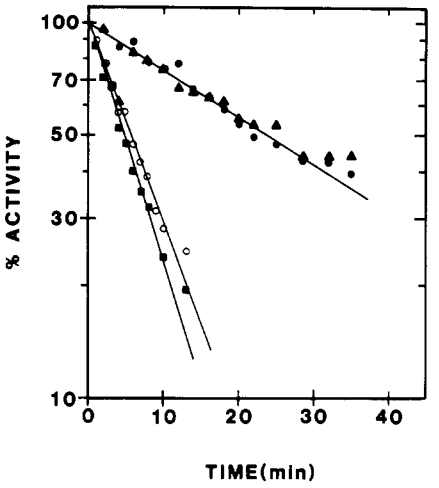

FIGURE 2: Inactivation of native and protected luciferase by the limited action of α-chymotrypsin. Reactions were carried out as in Figure 1, except that α-chymotrypsin (final concentration of 0.2 mg/mL) was used rather than *N*-ethylmaleimide. In the absence of protease, the luciferase activity was stable. Without flavin (■), the first-order rate constant for loss of luciferase activity was 0.11 min^{-1}. In the presence of 24 μM FMN [plus H_2O_2 (○)], the rate constant was 0.092 min^{-1}, only slightly slower than that without flavin. However, when $FMNH_2$ was injected into the aerobic solution of luciferase (●), the rate constant for subsequent inactivation of the luciferase by α-chymotrypsin was decreased to ca. 0.029 min^{-1}. To determine whether the flavin might interfere with the action of the protease, the luciferase was removed from the flavin by chromatography on Sephadex G-25 as described in the text. A solution of 260 μL of luciferase (0.1 mM) and FMN (0.55 mM) was reduced with an excess of sodium dithionite and applied to the chromatography column. The eluted luciferase was adjusted to 1.7 μM with buffer and the reaction started by addition of α-chymotrypsin (0.2 mg/mL final concentration). The rate of inactivation was 0.029 min^{-1} (▲), indistinguishable from the rate of inactivation in the presence of excess flavin (●).

was very similar to that of Figure 1, except that α-chymotrypsin was used rather than *N*-ethylmaleimide. The protected form of the enzyme showed a striking reduction in sensitivity to α-chymotrypsin, just as the experiment of Figure 1 demonstrated a protection from *N*-ethylmaleimide. In this experiment, we also resolved the enzyme from flavin species to preclude the possible effects of flavin on the activity of α-chymotrypsin and to determine whether flavin remained bound (Figure 2, ▲). The luciferase–FMN mixture was reduced with dithionite and applied to a column of Sephadex G-25 equilibrated with 0.01% (v/v) *n*-decanal and 350 mM phosphate, pH 7.0, at 1 °C. Immediately following elution from the column (ca. 5 min), α-chymotrypsin was added to the luciferase sample and the rate of inactivation determined. The enzyme showed a striking protection from the protease comparable in magnitude to the protection from *N*-ethylmaleimide described by Ziegler-Nicoli et al. (1974) and again demonstrated in Figure 1. The data presented in Figure 2 also demonstrate that the enzyme is afforded only slight protection by the FMN resulting from autoxidation, less than that observed by Ziegler-Nicoli et al. (1974), probably due to the much higher phosphate concentration in the present experiments.

The chromatographic conditions used in these experiments were designed to determine whether the protected form of the enzyme observed by Ziegler-Nicoli et al. (1974) resulted from a slow relaxation of the enzyme or from a slow release of oxidized flavin. If the protected form of the enzyme resulted from bound oxidized flavin, the enzyme eluting from the column should be complexed with flavin. The amount of flavin eluting with the luciferase was determined by both absorbance

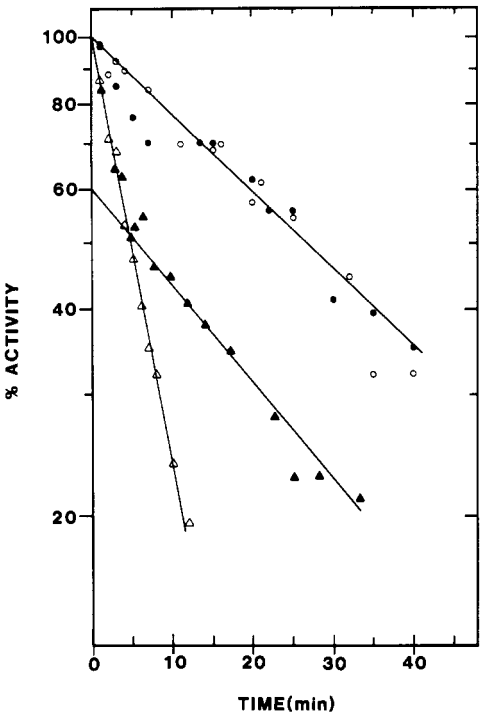

FIGURE 3: Conversion of the protected form of luciferase to the native form demonstrated by inactivation by α-chymotrypsin. The protected luciferase was prepared and resolved from flavin chromatographically as described in the legend to Figure 2. The eluted protein fraction was collected and either mixed immediately (at 0 time) with α-chymotrypsin [final concentration 0.1 (●) or 0.2 mg/mL (○)] or permitted to stand at 0 °C for 20 min prior to addition (at 0 time) of α-chymotrypsin [final concentration 0.2 mg/mL (▲)]. Final concentration of protected luciferase was 8.5 μM. Native enzyme (10 μM) in the column elution buffer was also inactivated by α-chymotrypsin [final concentration 0.2 mg/mL (△)]. First-order rate constants were 0.146 min^{-1} for the native enzyme (△), 0.026 min^{-1} for the protected enzyme at both protease concentrations (● and ○), and 0.146 and 0.032 min^{-1} for the fast and slow phases of the inactivation of the protected luciferase after 20-min incubation at 0 °C (▲).

and fluorescence spectroscopy. In no case was the luciferase:flavin ratio less than 2.5:1, and it was usually greater than 4:1. However, the luciferase showed a single-exponential inactivation by *N*-ethylmaleimide or by α-chymotrypsin if the inactivating agent was added immediately after the enzyme eluted from the column, even though there was substantially less than 1 mol of flavin per mole of enzyme.

In a related experiment, the rate of decay of the 4a-hydroxyflavin and the rate of appearance of free FMN were measured fluorometrically. Protected enzyme eluted from the column was diluted to 1.3 μM enzyme with 9 °C column elution buffer, and fluorescence measurements were begun immediately at 9 °C. The decay of 4a-hydroxyflavin was measured with excitation at 380 nm and emission at 490 nm, as described by Kurfurst et al. (1984). Free flavin was monitored by excitation at 380 nm and emission at 535 nm. Enzyme-bound FMN is nonfluorescent (Baldwin et al., 1975). Both reactions appeared to be first order and to have the same rate constant (0.14 min^{-1}) at 9 °C. This observation supports the conclusion that the protection of the less susceptible form of the enzyme is not due to bound flavin.

To determine whether the mixture of protected and sensitive forms of the enzyme evident in the experiment of Figure 1 could be detected by the protease-sensitivity assay, we performed another experiment similar to that of Figure 1 using α-chymotrypsin rather than *N*-ethylmaleimide. The results of this experiment are presented in Figure 3. Enzyme remaining after decay of intermediate II was removed from flavin species as in Figure 2. Addition of α-chymotrypsin to the enzyme immediately after elution from the column resulted in inactivation of the enzyme at a rate about 20% that of the control enzyme. Enzyme that was incubated at 0 °C for 20 min after elution from the column showed a biphasic inactivation with α-chymotrypsin, similar to that observed with *N*-ethylmaleimide and shown in Figure 1. The initial phase of the inactivation was comparable, within experimental error, to that of the control, and the second phase was similar to that of the protected form of the enzyme observed when the protease was added immediately after resolution from the flavin. The slow phase was not subtracted from the initial phase to determine the true rate of inactivation due to the imprecision of the data at the early times of reaction. While this lack of correction does lead to inaccuracy in the rate constants, the error does not alter the conclusion to be drawn from the experiment.

To determine whether the protected form of the enzyme was being inactivated, albeit at a slower rate than the control enzyme, or the observed rate of inactivation by α-chymotrypsin was limited by the relaxation of the protected conformation to that of the control enzyme, the concentration of the protease was varied (Figure 3). There was no measurable change in the rate of inactivation with a 2-fold change in the protease concentration, indicating that the rate-limiting step in the inactivation of the luciferase under the conditions of this experiment was the relaxation of the luciferase to the susceptibile conformation, not interaction between the protease and the luciferase.

To further investigate the question of whether the rate-limiting step in the proteolytic inactivation of the protected form of the luciferase might be the relaxation step, the effect of protease concentration on the rate of inactivation of the protected luciferase and the luciferase in the presence of FMN and H_2O_2 was studied (Figure 4). In this experiment, the protected form of the luciferase was prepared as described in Figure 1 and was not resolved from the flavin. The rate of inactivation of the protected form of the luciferase appeared to be zero order with respect to protease over a 4-fold range of protease concentrations (0.1 mg/mL in Figure 3 to 0.4 mg/mL in Figure 4), while the inactivation of the native enzyme in the presence of FMN and H_2O_2 appeared to be first order with respect to protease concentration. The first-order dependence of the inactivation of luciferase by proteases has been a much-studied and exploited property of the reaction (Njus et al., 1974; Baldwin, 1978). Never before have we found conditions in which the inactivation of luciferase did not show a first-order dependence on protease concentration.

DISCUSSION

The experiments reported here suggested that luciferase was in an altered conformational state following a single cycle of intermediate II formation and decay. This altered conformational state appeared to relax to the original state with a half-time of ca. 25 min at 0 °C and was characterized by having a decreased sensitivity to inactivation by *N*-ethylmaleimide and by α-chymotrypsin. If the protease or the alkylating agent were added to the enzyme prior to complete conversion to the original susceptible form, both forms were readily apparent due to the dramatic differences in the rates of inactivation of the two forms. Under the conditions of our experiments, the rate-determining step for the conversion to the original sensitive form appeared to be a first-order conformational change in the enzyme that proceeded more slowly than the release of the product FMN.

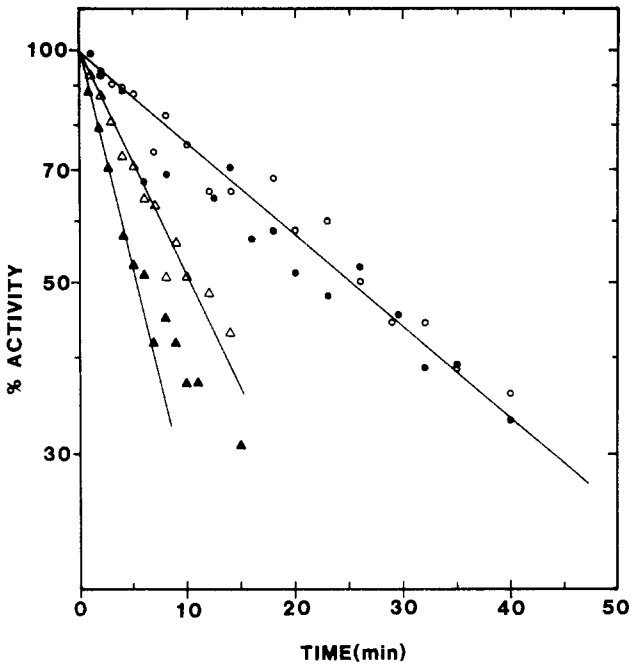

FIGURE 4: Effect of protease concentration on the rate of inactivation of native and protected forms of the luciferase. Samples of native and protected luciferase were prepared as described in the legend to Figure 1. Reactions were carried out in 350 mM phosphate, pH 7.0, at 0 °C. Final reaction concentrations were the following: luciferase, 10 μM; chymotrypsin, 0.2 mg/mL (open symbols) or 0.4 mg/mL (closed symbols); flavin, 25 μM. The first-order rate constants were 0.028 min^{-1} for the protected luciferase with both concentrations of protease (circles, compared with 0.026 min^{-1} for the similar experiment in Figure 3), 0.069 min^{-1} with 0.2 mg/mL protease in the presence of FMN [and H_2O_2 (△) compared with 0.146 min^{-1} for the reaction in the absence of FMN in Figure 3], and 0.139 min^{-1} with 0.4 mg/mL protease (▲).

The formation of the protected conformation did not appear to require complete cycling through the bioluminescence reaction; reaction of the enzyme-bound $FMNH_2$ with O_2 appeared to be sufficient to generate the protected state. The complete bioluminescence reaction that was carried out on the chromatography column apparently resulted in generation of the same conformational state as generation of intermediate II in the absence of aldehyde (see Figure 5). The protected form of the enzyme (E′ in Figure 5) appears to relax to the original sensitive form of the enzyme with the same rate, within experimental error, regardless of whether it is formed from intermediate II or by the complete bioluminescence reaction (see Figures 2–4).

It is interesting to note that the protected conformation of the enzyme has a decreased reactivity with alkylating reagents that modify the reactive thiol (Ziegler-Nicoli et al., 1974) and with ethoxyformic anhydride, which has been shown to modify an essential histidinyl residue on the α subunit (Cousineau & Meighen, 1976), but has essentially the same reactivity as the native enzyme with 2,4-dinitrofluorobenzene, which has been shown to react rapidly and specifically with the α-amino groups of both subunits (Welches & Baldwin, 1981). Modification of luciferase with 2,4-dinitrofluorobenzene leads to loss of measurable binding of $FMNH_2$, and binding of FMN protects the enzyme from the reagent (Welches & Baldwin, 1981). These observations are consistent with the conclusion reached here that the protected form of the enzyme does not have FMN bound. It appears that the environment of the α-amino groups modified by 2,4-dinitrofluorobenzene is not significantly different in the protected state, while the environments of the essential cysteinyl residue, the essential histidinyl residue, and

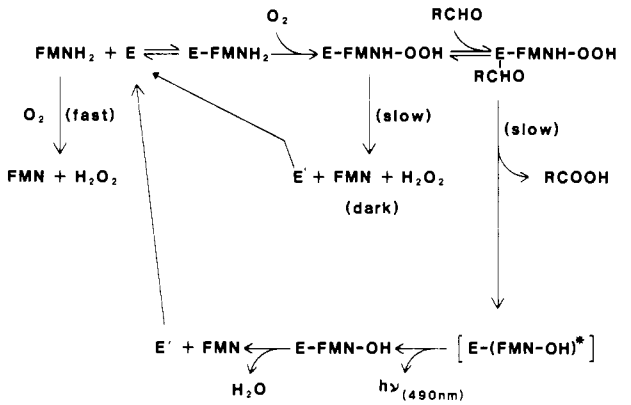

FIGURE 5: Proposed reaction pathway for the bacterial bioluminescence reaction. The scheme is a modification of that presented by Kurfurst et al. (1984) to accommodate the slow conformational changes in the enzyme reported here. For simplicity, the scheme does not include the random order of binding of aldehyde reported by Holzman & Baldwin (1983); for accuracy, this point is critical.

the bonds that are exposed to α-chymotrypsin in the native enzyme must be sufficiently different in the protected state to reduce the rates of the respective reactions. We cannot say from these results whether the decreased reactivity of the reactive cysteinyl or histidinyl residues is caused by decreased accessibility to the reagents or by an altered reactivity due to, for example, an increased pK_a.

The biphasic inactivation kinetics are indicative of a mixture of (at least) two species of luciferase in the reaction mixtures. The proportion of the two species changes with time, indicating that the two are interconvertible. We cannot from these results comment on the possibility that two different kinds of luciferase molecules are present from the outset, but we can state that if two types of luciferase molecules exist, they cannot be distinguished on the basis of their reactivity with alkylating reagents or sensitivity to proteases, as are the conformational states described here.

We have no information at this time regarding the possible significance of the protected form of the enzyme to its biological function in the cell. Luciferase is unique among flavoproteins in that, at least in vitro, the oxidized flavin dissociates, is reduced by a flavin oxidoreductase, and rebinds to the enzyme as the reduced coenzyme. It is possible that this altered conformational state is critical for proper functioning of the enzyme in vivo, either for productive interaction with another enzyme or enzymes in some sort of complex or for high-affinity binding of the low level of $FMNH_2$ found free in the cell. The results obtained here suggest that further study of the protected form of the enzyme to determine such parameters as $FMNH_2$ binding affinity and possible interactions of the protected form with other enzymes in the cell should provide insight into the luciferase system.

Registry No. $FMNH_2$, 5666-16-0; FMN, 146-17-8; *N*-ethylmaleimide, 128-53-0; α-chymotrypsin, 9004-07-3; luciferase, 9014-00-0.

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Chemical Trapping of Complexes of Dihydroxyacetone Phosphate with Muscle Fructose-1,6-bisphosphate Aldolase†

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ABSTRACT: Dihydroxyacetone phosphate (DHAP) in equilibrium with FDP aldolase of muscle is present in the form of two major covalent complexes. One, representing ~60% of total bound substrate, decomposes to P_i and methylglyoxal upon acid denaturation of the enzyme as first reported by Grazi and Trombetta [Grazi, E., & Trombetta, G. (1979) *Biochem. J. 175*, 361–365]. This is now shown to be the enzyme–eneamine phosphate reaction intermediate since P_i formation is prevented if the acid denaturation is done in the presence of potassium ferricyanide, an oxidant of the eneamine. The enzyme–eneamine aldehyde·P_i **6**, presumed to be an intermediate of the slow methylglyoxal synthetase reaction of aldolase, must not be a significant source of the P_i produced upon denaturation and is probably not a significant component of the equilibrium. The oxidation product, the enzyme–imine of phosphopyruvaldehyde, is sufficiently stable in 1 N HCl, $t_{1/2}$ = 76 min at 0 °C, to be isolated with the trichloroacetic acid precipitated protein. A second covalent complex, ~20–24% of bound dihydroxyacetone [^{32}P]phosphate, remains with the protein during acid denaturation and centrifugation. This acid-stable complex is formed rapidly and is chased rapidly by unlabeled substrate. Its stability in 1 N HCl is similar to that of the ferricyanide-oxidized derivative mentioned above. From this and its reactivity with cyanoborohydride in acid, this complex is thought to be the imine adduct of DHAP with aldolase **4** and/or the carbinolamine complex **3** present in the initial equilibrium. D-Glyceraldehyde 3-phosphate in the carbonyl form also forms an acid-precipitable complex with aldolase. However, its dissociation from the enzyme under conditions usual for studying the aldolase reaction is slow, $t_{1/2} > 20$ s at 25 °C, so that the complex must not be functional. The ability to trap and isolate covalent complexes in the Schiff-base class of enzymes offers a new and versatile approach to the study of these reaction intermediates in the steady state and at equilibrium and to explaining more precisely the consequences to the catalytic process of protein modification, solution pH, and alteration in substrate structure.

The sequence for interaction of aldolase and dehydroxyacetone phosphate (DHAP)[1] shown in Scheme I for steps prior to reaction with aldehyde is strongly indicated by several arguments: (1) proton exchange with water is catalyzed in the absence of aldehyde (Rieder & Rose, 1955), (2) reductive inactivation by $NaBH_4$ in the presence of excess DHAP is stoichiometric giving the secondary amine as expected for an imine with a unique N^{ϵ}-lysine (Grazi et al., 1963), (3) exchange of the C2 oxygen of substrate with H_2O is at least as rapid as aldol cleavage (Model et al., 1968), and (4) oxidants such as $K_3Fe(CN)_6$ are cosubstrates for the catalytic oxidation of DHAP by way of the eneamine **5** to pyruvaldehyde 3-phosphate (Healy & Christen, 1973).

We wished to examine the possibility that the imine and carbinolamine intermediates **4** may be sufficiently stable in acid to survive as covalent complexes with acid-denatured enzyme that can be brought down by centrifugation. This possibility is based on the expectation that (1) for DHAP to be released from the imine complex **4** it must pass through the zwitterion state, (2) acid conditions would favor the protonated carbinolamine, pK_a = 8–9, slowing the release, and (3) dehydration of the carbinolamine to the imine is acid catalyzed and favored in aqueous solution (Jencks, 1969; Hine

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[1] Abbreviations: BH_3, sodium cyanoborohydride; DHAP, dihydroxyacetone phosphate; FDP, fructose 1,6-bisphosphate; G3P, glyceraldehyde 3-phosphate; MG, methylglyoxal; TCA, trichloroacetic acid.

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