Identification of a Firefly Luciferase Active Site Peptide Using a Benzophenone-based Photooxidation Reagent*

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Bruce R. Branchini‡§, Rachelle A. Magyar‡, Karen M. Marcantonio‡, Kate J. Newberry‡, Justin G. Stroh®, Linda K. Hinz®, and Martha H. Murtiashaw‡

From the ‡Department of Chemistry, Connecticut College, New London, Connecticut 06320 and ¶Central Research Division, Pfizer Inc., Groton, Connecticut 06340

Firefly luciferase catalyzes the highly efficient emission of yellow-green light from substrate luciferin by a series of reactions that require MgATP and molecular oxygen. We prepared 2-(4-benzoylphenyl)thiazole-4-carboxylic acid (BPTC), a novel benzophenone-based substrate analog, intending to use it in photoaffinity labeling studies to probe the luciferase active site. Instead, we found that while BPTC was a potent photoinactivating reagent for firefly luciferase, it was not a photoaffinity labeling agent. Using proteolysis, reverse phase high-performance liquid chromatography, tandem high performance liquid chromatography-electrospray ionization mass spectrometry, and Edman sequencing, we identified a single luciferase peptide, 244HHGF247, the degradation of which was directly correlated to luciferase photoinactivation. Results of enzyme kinetics and related studies were consistent with this peptide being at or near the luciferin binding site. Further, peptide model studies and additional investigations on the nature of the photoinactivation process strongly suggested that BPTC catalyzed the formation of singlet oxygen at the active site of the enzyme. We describe here an uncommon example of active site-directed photooxidation of an enzyme by singlet oxygen.

Bioluminescence is a captivating process in which living organisms convert chemical energy into light. Beetle bioluminescence, including perhaps 2000 species of fireflies (1, 2), has fascinated scientists for many years. Beetle bioluminescence provides the basis for a wide variety of biochemical assays, many with important clinical applications (4, 5). Moreover, the luciferase gene (6) is widely used as a reporter in studies of gene expression and regulation (7). Additionally, the cloning and sequencing of P. pyralis luciferase and homologues from several beetles (8) have revealed that these enzymes are closely related to a large family of nonbioluminescent proteins (9, 10) that catalyze reactions of ATP with carboxylate substrates to form acyl adenylates.

The recent solving of the crystal structure of luciferase without bound substrates or ligands revealed an apparently unique molecular architecture consisting of two distinct domains (11); however, a detailed description of the luciferase active site remains elusive. Early chemical modification studies have failed to document the involvement of any specific amino acid residues in substrate binding or enzyme catalysis. Many of these studies have been hampered by the high nucleophilic reactivity of several of the luciferase cysteine sulfhydryl groups. However, the results of mutagenesis and other studies (12–14) substantiate the nonessential role of the luciferase cysteines. Additional mutational studies (8, 15–17) have identified individual amino acids and regions of the protein, the modification of which alters the color of bioluminescence. No direct structure-function relationship has emerged to account for the color variation of firefly bioluminescence. We have recently used mutational analysis directed at the structural motif common to the superfamily of adenylation-forming enzymes to demonstrate that Ser-198 is directly involved in determining the color of bioluminescence. No direct structure-function relationship has emerged to account for the color variation of firefly bioluminescence. We have recently used mutational analysis directed at the structural motif common to the superfamily of adenylation-forming enzymes to demonstrate that Ser-198 is directly involved in determining the color of bioluminescence. No direct structure-function relationship has emerged to account for the color variation of firefly bioluminescence. We have recently used mutational analysis directed at the structural motif common to the superfamily of adenylation-forming enzymes to demonstrate that Ser-198 is directly involved in determining the color of bioluminescence. No direct structure-function relationship has emerged to account for the color variation of firefly bioluminescence. We have recently used mutational analysis directed at the structural motif common to the superfamily of adenylation-forming enzymes to demonstrate that Ser-198 is directly involved in determining the color of bioluminescence. No direct structure-function relationship has emerged to account for the color variation of firefly bioluminescence. We have recently used mutational analysis directed at the structural motif common to the superfamily of adenylation-forming enzymes to demonstrate that Ser-198 is directly involved in determining the color of bioluminescence. No direct structure-function relationship has emerged to account for the color variation of firefly bioluminescence. We have recently used mutational analysis directed at the structural motif common to the superfamily of adenylation-forming enzymes to demonstrate that Ser-198 is directly involved in determining the color of bioluminescence. No direct structure-function relationship has emerged to account for the color variation of firefly bioluminescence. We have recently used mutational analysis directed at the structural motif common to the superfamily of adenylation-forming enzymes to demonstrate that Ser-198 is directly involved in determining the color of bioluminescence. No direct structure-function relationship has emerged to account for the color variation of firefly bioluminescence. We have recently used mutational analysis directed at the structural motif common to the superfamily of adenylation-forming enzymes to demonstrate that Ser-198 is directly involved in determining the color of bioluminescence. No direct structure-function relationship has emerged to account for the color variation of firefly bioluminescence. We have recently used mutational analysis directed at the structural motif common to the superfamily of adenylation-forming enzymes to demonstrate that Ser-198 is directly involved in determining the color of bioluminescence. No direct structure-function relationship has emerged to account for the color variation of firefly bioluminescence. We have recently used mutational analysis directed at the structural motif common to the superfamily of adenylation-forming enzymes to demonstrate that Ser-198 is directly involved in determining the color of bioluminescence. No direct structure-function relationship has emerged to account for the color variation of firefly bioluminescence. We have recently used mutational analysis directed at the structural motif common to the superfamily of adenylation-forming enzymes to demonstrate that Ser-198 is directly involved in determining the color of bioluminescence. No direct structure-function relationship has emerged to account for the color variation of firefly bioluminescence.

In this study, we initially focused on characterizing the firefly luciferin binding site. We prepared BPTC, a novel benzophenone-based dehydroluciferin analog (Fig. 1). Dehydroluciferin is a potent reversible luciferase inhibitor (18) and a nonbioluminescent substrate for the adenylation reaction (19). We intended to use BPTC as a photoaffinity labeling reagent, fully expecting the benzophenone photophore to demonstrate that Ser-198 is directly involved in determining the color of bioluminescence. No direct structure-function relationship has emerged to account for the color variation of firefly bioluminescence. We have recently used mutational analysis directed at the structural motif common to the superfamily of adenylation-forming enzymes to demonstrate that Ser-198 is directly involved in determining the color of bioluminescence. No direct structure-function relationship has emerged to account for the color variation of firefly bioluminescence. 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ousley investigated without success.\(^3\) We report here that BPTC is a potent photoactivating reagent for firefly luciferase, but not a photoaffinity labeling agent. Instead, we will show that BPTC is a site-specific photoxidizing reagent that has enabled us to identify a luciferase active site peptide.

**EXPERIMENTAL PROCEDURES**

**Materials**—The following items were obtained from the indicated sources: firefly luciferase (crystallized and lyophilized powder from *Photinus pyralis*, EC 1.13.12.7), MgATP (equine muscle), N\(^\text{\textast}}\)p-toyyl-L-lysine chloromethyl ketone-treated chymotrypsin and sequencing grade trifluoracetic acid (Sigma); HHGF (Macromolecular Resources). 4-Cyano-benzophenone (20) was prepared in two steps from 4-cyano-benzaldehyde and phenylmagnesium bromide by the procedure of Wagner and Siebert (21). HOPhHA was synthesized by a literature method (22).

**Synthesis and Characterization of BPTC**—4-Benzoylphenylthiocarboxamide—Hydrogen sulfide was bubbled for 2 h at room temperature into a stirred solution of 4-cyano-benzophenone (400 mg, 1.9 mmol) in anhydrous pyridine (2.5 ml) containing a few drops of triethylamine. Nitrogen then was bubbled through the solution to remove H\(_2\)S, and the mixture was rotary evaporated leaving a yellow solid (400 mg, 1.7 mmol, 87%). The crude product was purified by flash chromatography (silica gel, acetone:hexanes, 30:70, v/v) to a yellow solid (400 mg, 1.7 mmol, 87%). The crude product was purified by flash chromatography (silica gel, acetone:hexanes, 30:70, v/v) to a yellow solid (400 mg, 1.7 mmol, 87%).

**Identification of a Firefly Luciferase Active Site Peptide**

**BPTC** is a site-specific photooxidizing reagent that has enabled us to identify a luciferase active site peptide.

**Luciferase Photoinactivation Studies**—Luciferase (0.5–0.6 mg/ml) in NaPB was mixed with BPTC (0.8–100 \(\mu\)M) and solutions were irradiated with a Rayonet 350 lamp (emitted light, 300–400 nm) at a distance of 7 cm. During photolysis, all samples were kept on ice under a Pyrex glass filter. In the standard photoinactivation protocol, no special precautions were made to remove O\(_2\). To exclude O\(_2\), NaPB solutions were bubbled with argon for 20 min and, in some cases, sodium dithionite (12.5 mm) was included.

**Kinetic Constants**—The reversible inhibition of luciferase by BPTC and HOPhHA was assessed with respect to luciferin and MgATP. For luciferin (6–40 \(\mu\)M), BPTC and HOPhHA levels ranged from 2.5 to 20 \(\mu\)M and 70 to 700 \(\mu\)M, respectively. For substrate MgATP (4–325 \(\mu\)M), BPTC and HOPhHA concentrations were 0.04–13.0 \(\mu\)M and 20–80 \(\mu\)M, respectively. Inhibitory constants (\(K_I\)) were estimated by linear least squares fits of data from initial velocity measurements performed in the dark. The data with each luciferase substrate were analyzed by the method of Dixon (24), and the \(K_I\) for MgATP was obtained using the method of Cornish-Bowden for uncompetitive inhibitors. An independent estimate of the BPTC binding constant was made from the irreversible photoinactivation data in Fig. 2. A plot of \(1/\text{obsd} V_a / \text{obsd} V_{A0} = 1/BPTC\) was used to determine \(K_2\) and \(K_3\) according to the equation \(1/\text{obsd} V_a / \text{obsd} V_{A0} = K_2/K_3[BPTC] + 1/K_3\).

**Protolytic and Analysis of Luciferase Peptides**—BPTC-inactivated luciferase (5% activity) and control enzyme preparations (irradiated and dark) were digested with N\(^\text{\textast}}\)p-toyyl-L-lysine chloromethyl ketone-treated chymotrypsin (protease-protein, 1:20, w/w) in NaPB for 2 h at 25 \(\pm\) 1°C and then quenched by addition of soybean trypsin inhibitor. Chymotryptic peptides were separated by RP-HPLC (14); specific conditions are detailed in the figure legends. The peak associated with BPTC photoinactivation was collected from control and inhibited luciferase preparations (Fig. 3), concentrated by lyophilization, purified by rechromatography, and analyzed by LC/ESMS (14) and N-terminal sequencing (Biotechnology Support Facility, University of Kansas Medical Center).

**Model Photooxidation Studies**—Solutions of peptide HHGF, BPTC, and various additives were irradiated in NaPB at room temperature as described under “Luciferase Photoinactivation Studies.” Aliquots were withdrawn and analyzed by RP-HPLC (14). Elution conditions were 40 °C at a flow rate of 1 ml/min, using 0.1% aqueous TFA containing linear gradients of acetonitrile: 10% (v/v) for 5 min; 20% after 20 min; and 75% after 60 min. HHGF concentrations were determined by peak integration. In a separate experiment, the products from irradiation of HHGF (200 \(\mu\)M) in the presence of BPTC (400 \(\mu\)M) were analyzed by LC/ESMS.

**RESULTS**

**Synthesis and Characterization of BPTC**—BPTC (Fig. 1) was prepared in three steps in 33% overall yield from 4-cyano-benzophenone. The synthetic route was modeled after the preparation of dehydroluciferin developed by White et al. (25). The BPTC structure was confirmed by high resolution mass spectrometry, IR, UV-visible, \(^1\)H- and \(^{13}\)C-NMR spectroscopy (see “Experimental Procedures”). The high molar absorptivity of BPTC solutions in NaPB (\(\lambda_{max} 312 \text{ nm, } \epsilon = 4.31\)) enabled photolysis experiments to be carried out with a Rayonet 350 nm

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\(^3\) B. R. Branchini and M. H. Martiashaw, unpublished results.
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**BPTC**

found to be an uncompetitive inhibitor ($K_i$) luciferin ($I$), enzyme activity (14). Steady-state kinetic analysis (data not shown) of the effect of BPTC Inhibition of Luciferase Is Active Site Directed—Irreversible Photoinactivation of Luciferase by BPTC—Iradiation of solutions of native luciferase (8 $\mu$m) and BPTC (0.8–100 $\mu$m) effected a time- and concentration-dependent inactivation of bioluminescence activity (Fig. 2). Identical results were obtained when recombinant enzyme was irradiated with several concentrations of BPTC. Equimolar and higher concentrations of BPTC rapidly inactivated luciferase. Interestingly, substoichiometric levels of BPTC also could fully inhibit the enzyme. The failure of gel filtration, dialysis, and incubation with dithiothreitol to regenerate enzyme activity provided further evidence for the irreversible nature of the photoinactivation process (data not shown). Additional control studies (Table I) demonstrated that luciferase retained full activity when irradiated in the absence of BPTC or when incubated in the dark with BPTC, affirming that the photoinactivation of luciferase required BPTC.

**BPTC Inhibition of Luciferase Is Active Site Directed—Steady-state kinetic analysis (data not shown) of the effect of BPTC (without irradiation) on luciferase flash height generation demonstrated that BPTC was a very good reversible competitive inhibitor ($K_c = 3.0 \pm 0.3 \mu$m) with respect to substrate luciferin ($K_m = 15 \pm 4.0 \mu$m) (26). The measured $K_v$ value for BPTC is similar to that reported (18) for dehydrroluciferin ($K_v = 1.0 \mu$m), the structure BPTC was designed to resemble. With respect to substrate MgATP ($K_m = 110 \mu$m) (27), BPTC was found to be an uncompetitive inhibitor ($K_v = 0.55 \pm 0.05 \mu$m). The BPTC $K_v$ value was independently estimated to be 7.5 $\mu$m from analysis of the irreversible inactivation data presented in Fig. 2 (insert). Attempts to determine whether luciferin or known analogs could protect against BPTC photoinactivation were unsuccessful because these compounds absorb strongly in the irradiated region. To circumvent this problem, we prepared HOPhHA (20 $\mu$m) and MgATP (10 $\mu$m) BPTC. Peptides (~75 $\mu$g) were separated at 40 °C by RP-HPLC on a Rainin C$_{18}$ 5-$\mu$m column (4.6 × 250 mm) eluted at a flow rate of 1 ml/min, using 0.1% aqueous TFA containing linear gradients of acetonitrile: 5% (v/v) for 5 min; 15% after 10 min; 25% after 20 min; 50% after 45 min; and 95% after 60 min. Inset, the designated (*) peaks from 250 $\mu$m of chymotrypsin digests of control and BPTC-photoinactivated luciferase samples were collected, lyophilized, and rechromatographed using 0.1% aqueous TFA with a linear gradient of acetonitrile: 10% (v/v) for 5 min; 12% after 10 min; 20% after 36 min; 90% after 56 min. The indicated peaks (#) for the control (upper trace) and photoinactivated samples (lower trace) were collected and analyzed by LC/ESMS (14) and Edman sequencing.

![Plot](Image)

**Fig. 2. Photoactivity of luciferase by BPTC.** Luciferase (8 $\mu$m) was irradiated at 4 °C in NaPB in the absence (●) or presence of BPTC: 100 $\mu$m; ○, 8 $\mu$m; △, 4 $\mu$m; ×, 2.8 $\mu$m; ◊, 0.8 $\mu$m. At the indicated times, 4-$\mu$l aliquots were withdrawn and analyzed for enzyme activity as described under “Experimental Procedures.” Prolonged irradiation of BPTC up to 2 h did not alter its absorption spectrum. In the dark, using 0.1 mM BPTC, 6.0 ± 1.0 nmol of pyrophosphate/min/mg luciferase were released at pH 8.6. Thus, BPTC was a substrate for the luciferase-catalyzed adenylation reaction at pH 8.6 (although not at pH 7.8) as shown in Equation 1.

**Irreversible Photoinactivation of Luciferase by BPTC—**

\[ I + BPTC \rightarrow (I \cdot BPTC) \quad (1) \]

To determine whether luciferin or known analogs could protect against BPTC photoinactivation we prepared HOPhHA (20 $\mu$m) and MgATP (10 $\mu$m) BPTC. Peptides (~75 $\mu$g) were separated at 40 °C by RP-HPLC on a Rainin C$_{18}$ 5-$\mu$m column (4.6 × 250 mm) eluted at a flow rate of 1 ml/min, using 0.1% aqueous TFA containing linear gradients of acetonitrile: 5% (v/v) for 5 min; 15% after 10 min; 25% after 20 min; 50% after 45 min; and 95% after 60 min. Inset, the designated (*) peaks from 250 $\mu$m of chymotrypsin digests of control and BPTC-photoinactivated luciferase samples were collected, lyophilized, and rechromatographed using 0.1% aqueous TFA with a linear gradient of acetonitrile: 10% (v/v) for 5 min; 12% after 10 min; 20% after 36 min; 90% after 56 min. The indicated peaks (#) for the control (upper trace) and photoinactivated samples (lower trace) were collected and analyzed by LC/ESMS (14) and Edman sequencing.

![RP-HPLC separation of control and BPTC-inactivated luciferase peptides.](Image)

**Fig. 3. RP-HPLC separation of control and BPTC-inactivated luciferase peptides.** Chymotryptic digests were prepared from luciferase (8 $\mu$m) irradiated in NaPB without (upper trace) and with lower (lower trace) 4 $\mu$m BPTC. Peptides (~75 $\mu$g) were separated at 40 °C by RP-HPLC on a Rainin C$_{18}$ 5-$\mu$m column (4.6 × 250 mm) eluted at a flow rate of 1 ml/min, using 0.1% aqueous TFA containing linear gradients of acetonitrile: 5% (v/v) for 5 min; 15% after 10 min; 25% after 20 min; 50% after 45 min; and 95% after 60 min. Inset, the designated (*) peaks from 250 $\mu$m of chymotrypsin digests of control and BPTC-photoinactivated luciferase samples were collected, lyophilized, and rechromatographed using 0.1% aqueous TFA with a linear gradient of acetonitrile: 10% (v/v) for 5 min; 12% after 10 min; 20% after 36 min; 90% after 56 min. The indicated peaks (#) for the control (upper trace) and photoinactivated samples (lower trace) were collected and analyzed by LC/ESMS (14) and Edman sequencing.

![Graph](Image)

**Table I**

| Additions/conditions | Half-life $b$ | % loss of activity $b$ |
|---------------------|--------------|------------------------|
| BPTC omitted        | 0            | 0                      |
| No irradiation      | 0            | 0                      |
| None                | 7.5          | 98                     |
| HOPHHA (20 $\mu$m)  | 75.0         | 25                     |
| MgATP (10 $\mu$m)   | 30.1         | 55                     |
| Argon bubbling      | 14.3         | 84                     |
| Sodium dithionite (2.5 mm) | 301.0 | 7                   |
| NaPB-D$_2$O (96%)   | 4.4          | 99                     |
| Superoxide dismutant (1 unit/ml) | 7.5 | 98                 |
| Mannitol (10 mm)    | 7.5          | 98                     |
| Sodium azide (10 mm) | 10.0         | 94                     |

$^a$ Reaction mixtures containing 8 $\mu$m luciferase and 4 $\mu$m BPTC in NaPB were irradiated for 40 min as described under “Experimental Procedures.”

$^b$ Half-lives were calculated from rates measured by monitoring loss of enzyme activity as described under “Experimental Procedures.”
Identification of a Firefly Luciferase Active Site Peptide—RP-HPLC was used to separate proteolytic fragments generated from control and photoinactivated luciferase digested with chymotrypsin, trypsin, or endoproteinase Glu-C. Only chymotrypsin digests presented a pattern of peptides with any differences between control and inactivated samples, the sole difference being a ~75% decrease in the area of the peak indicated by an asterisk in Fig. 3. The decrease in area of this peak was directly correlated with the loss of enzyme activity (data not shown). However, no new peaks appeared in the digest of inactivated protein.

The peaks designated in Fig. 3 were further purified by rechromatography (Fig. 3, inset). By Edman sequencing and LC/ESMS, both peaks were found to contain two coeluting peptides, HHGF (MH+ = 497.4) and VRGPM (MH+ = 559.4), corresponding to regions 244–247 and 392–396 in the deduced luciferase sequence (6). The mole percentage of HHGF:VRGPM in the peak from the control sample was estimated from the sequencing data to be 78:22. In the photoinactivated sample, the HHGF content decreased ~70%, while the amount of VRGPM remained unchanged. Therefore, the loss of peptide 244HHGF247 was correlated to the BPTC-mediated photoinactivation process.

Model Photooxidation Studies with HHGF.—The absence of any evidence for BPTC-peptide adduct formation suggested that no true photoaffinity labeling had occurred during the photoinactivation process. Instead, it seemed likely that photooxidation of one or both of the imidazole side chains of 244HHGF247 may be a major cause of luciferase inactivation.

A model study was undertaken in which synthetic HHGF (200 µM) and BPTC (400 µM) in NaPB were irradiated at room temperature for 1 h. A portion (35 µl) of the mixture was analyzed by LC/ESMS (14), and the RP-HPLC chromatogram is shown. Inset, calculated and observed masses (MH+) for the putative products are tabulated. No assignable mass data were obtained for peaks 2 and 6, and peaks 8–10 corresponded to known system contaminants.

BPTC Photooxidizes Luciferase—The results of luciferase photoinactivation experiments in which soluble oxygen was reduced by argon bubbling and sodium dithionite indicated decreased or strongly suppressed enzyme inhibition (Table I). A control trial showed that sodium dithionite had no effect on BPTC stability. Therefore, unlike photoaffinity labeling experiments that are usually retarded by oxygen, BPTC-mediated photoinactivation required it. Since substoichiometric concentrations of BPTC completely inactivated luciferase (Fig. 2) and since BPTC was not consumed in the process (Fig. 3), the benzophenone derivative must function as a photooxidation catalyst. Although sodium azide only modestly protected luciferase against BPTC photooxidation, evidence supporting the involvement of singlet oxygen included (Table I): (i) a 1.7-fold decrease in the inhibition half-life measured in deuterium oxide buffers; and (ii) the failure of superoxide dismutase or mannitol to alter the inactivation rate.

Since Cys, Met, Tyr, Trp, and His are especially susceptible to photooxidation (31), we compared the content of these amino acids in irradiated control and BPTC-inactivated luciferase (Table III). A loss of His consistent with the determined level of 244HHGF247 photooxidation and a very minor decrease in Cys were the only differences noted (including all other amino acids not shown in Table III).
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DISCUSSION

Active Site-directed Photoinactivation of Luciferase—Molecular modeling was used to design BPTC, a dehydro-luciferin analog containing a benzophenone moiety (Fig. 1). BPTC very effectively inactivated luciferase irreversibly by a process requiring irradiation (Fig. 2). Furthermore, luciferase modification occurred at or near the luciferin binding site as: (i) BPTC was found to be a substrate for the adenylation reaction shown to luciferin; and (iii) the BPTC-promoted inactivation process was effectively retarded by the luciferin analog HOPhHA (Table I). BPTC also was shown to be an uncompetitive inhibitor ($K_i = 3.0 \pm 0.3 \mu M$) with respect to luciferin; and (iii) the BPTC-promoted inactivation process was effectively retarded by the luciferin analog HOPhHA (Table I). BPTC also was shown to be an uncompetitive inhibitor ($K_i = 0.55 \pm 0.05 \mu M$) with respect to another luciferase substrate, MgATP. Since MgATP provided modest protection against photoinactivation, a portion of the luciferase inactivation may occur at a second site, possibly an allesteric binding site for ATP (32). Using proteolysis, RP-HPLC, LC/ESMS, and Edman sequencing (Fig. 3), we identified a single luciferase peptide, 244HHGF247, the degradation of which was directly correlated to luciferase photoinactivation. However, since complete enzyme inhibition was accompanied by the loss of only $\sim 70$–75$\%$ of the peptide, the BPTC-mediated photoinactivation process must occur at more than one site, possibly a cysteine residue. While HHGF was identified as the major site of luciferase modification by comparison to controls, no modified peptides were found. As suggested by model studies (Fig. 4), several modified HHGF peptides probably were formed, but no single one was produced at a measurable level.

Nature of the Photoinactivation Process—The modification of luciferase at 244HHGF247 could not, as expected for a benzo-phenone derivative, be associated with photoaffinity labeling because: (i) the photoinactivation process required oxygen; (ii) substoichiometric amounts of BPTC fully inactivated luciferase; (iii) no BPTC-HHGF adduct was detected; and (iv) BPTC was not consumed during the photoinactivation process. Instead, BPTC is a catalyst for luciferase photooxidation. There are many examples of substituted aryl ketones functioning as triplet sensitizers (35). In the BPTC structure, the extended conjugation provided by the para-substituted thiazole ring must shift the key electronic transition from $\pi^* \rightarrow \pi$ to $\pi^* \rightarrow \pi^*$, thereby producing a molecule much less reactive to H abstraction required for photoaffinity labeling (19). The extended conjugation in the BPTC structure also may make it a more efficient triplet sensitizer.

Although it is difficult to distinguish between type II (singlet oxygen) and type I (electron transfer between an excited triplet state and a substrate molecule) photodynamic processes, we propose that BPTC generates singlet oxygen at the luciferase active site in very close proximity to the peptide 244HHGF247. The results of model studies undertaken with synthetic HHGF (Table II; Fig. 4) strongly support our contention that BPTC is a sensitizer for singlet oxygen production. Likewise, evidence consistent with BPTC-promoted singlet oxygen inactivation of luciferase was compiled (Table I). However, two significant differences were noted between the enzyme and model studies: (i) azide ion was much less effective in protecting the protein; and (ii) the enzyme inactivation process was $\sim 150$ times faster than the photooxidation of the model peptide. Previously, Seliger and co-workers reported (33) the inactivation of luciferase with singlet oxygen generated by irradiating an immobilized Rose Bengal catalyst. They determined that luciferase was $\sim 10$ times more sensitive to singlet oxygen than histidine. The even greater sensitivity of luciferase to BPTC-photooxidation than the actual peptide being modified very likely reflects the close proximity of both oxygen and BPTC to the 244HHGF247 region. The failure of sodium azide to retard luciferase photooxidation in our study presumably reflects the inability of the ion to reach the enzyme site of singlet oxygen generation.

Possible Functional Role of 244HHGF247—To explain the greater sensitivity of luciferase to singlet oxygen, Seliger proposed (33) that a histidine residue was located at a putative enzyme binding site for molecular oxygen. Mutagenesis studies support a similar proposal for the importance of a histidine residue in oxygen binding in the bioluminescent photoprotein aequorin (34). Possibly, in the firefly protein, the region 244HHGF247 is involved in binding oxygen for subsequent addition to the luceryl adenylate (Equation 2). BPTC binding near this site would be expected to make His-244 and/or His-245 the most susceptible residue(s) to photooxidation as we have observed. Alternatively, the tetrapeptide may be involved in luciferin binding. Interestingly, His-245 is conserved among all of the 13 known firefly luciferase sequences (8). Preliminary results of ongoing mutagenesis studies support an important functional role for His-245 since the mutant proteins H245Q and H245A showed altered light emission kinetics and $\sim 200$–$400$-fold lower relative activities, respectively, compared with wild-type luciferase. Furthermore, 244HHGF247 is located in the 226–250 region of the luciferase primary sequence in which minor changes produce dramatic shifts in the color of bioluminescence (8, 16). This region is part of the $\beta$-sheet B subdomain, which is located in the large N-terminal domain of the luciferase structure (11). It appears that the tetrapeptide is located near an internal cavity containing ordered water molecules. The cavity is in a surface groove close to several invariant residues that comprise the putative luciferase active site (11). When the x-ray coordinates become available, it will be possible to better determine the location of the 244HHGF247 in the luciferase three-dimensional structure.

Concluding Remarks—We have described here an uncommon example of active site-directed photooxidation of an enzyme by singlet oxygen. At least one other example of site-directed photooxidation has been reported (36), although the active species responsible for oxidation of a Cys residue in $\Delta3$-ketosteroid isomerase was not determined. The benzo-phenone group, so useful in photoaffinity labeling studies (19), has been used here to efficiently catalyze the photooxidation of a histidine-containing firefly luciferase peptide. Potentially, BPTC and other substituted benzophenones with extended conjugation may prove generally useful as site-directed photooxidation reagents. We are currently investigating this possibility and extending the ongoing mutation studies to better determine the functional role of 244HHGF247 in firefly bioluminescence.

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