Site-Directed Mutagenesis of Firefly Luciferase Active Site Amino Acids: A Proposed Model for Bioluminescence Color†

Bruce R. Branchini,* Rachelle A. Magyar, Martha H. Murtiashaw, Shannon M. Anderson, Lisa C. Helgerson, and Marc Zimmer

Department of Chemistry, Connecticut College, New London, Connecticut 06320

Received May 21, 1999; Revised Manuscript Received July 22, 1999

ABSTRACT: Under physiological conditions firefly luciferase catalyzes the highly efficient emission of yellow−green light from the substrates luciferin, Mg-ATP, and oxygen. In nature, bioluminescence emission by beetle luciferases is observed in colors ranging from green (∼530 nm) to red (∼635 nm), yet all known luciferases use the same luciferin substrate. In an earlier report [Branchini, B. R., Magyar, R. M., Murtiashaw, M. H., Anderson, S. M., and Zimmer, M. (1998) *Biochemistry 37*, 15311−15319], we described the effects of mutations at His245 on luciferase activity. In the context of molecular modeling results, we proposed that His245 is located at the luciferase active site. We noted too that the H245 mutants displayed red-shifted bioluminescent emission spectra. We report here the construction and purification of additional His245 mutants, as well as mutants at residues Lys529 and Thr343, all of which are stringently conserved in the beetle luciferase sequences. Analysis of specific activity and steady-state kinetic constants suggested that these residues are involved in luciferase catalysis and the productive binding of substrates. Bioluminescence emission spectroscopy studies indicated that point mutations at His245 and Thr343 produced luciferases that emitted light over the color range from green to red. The results of mutational and biochemical studies with luciferase reported here have enabled us to propose speculative mechanisms for color determination in firefly bioluminescence. An essential role for Thr343, the participation of His245 and Arg218, and the involvement of bound AMP are indicated.

Bioluminescence is an appealing process by which living organisms convert chemical energy into light. Beetle bioluminescence, observed in perhaps 2000 species of fireflies (*1, 2*), has provided a fruitful area of basic and applied research mainly focused on the North American firefly *Photinus pyralis* (*3*). In this firefly species, the luciferase-catalyzed light emission process is extremely efficient; in alkaline solution, nearly a single photon is emitted per reacted luciferin molecule (*4*). The enzyme firefly luciferase functions as a monooxygenase, without the apparent involvement of a metal or cofactor.

According to the enzymatic mechanism (*5, 6*) presented in Figure 1, the beetle luciferases catalyze a sequence of reactions that convert firefly luciferin into an electronically excited state oxyluciferin product which then emits light. First, firefly luciferase catalyzes the formation of an enzyme-bound luciferyl adenylate (Figure 1, step a). Next, a proton is abstracted from the C-4 carbon of the adenylate by a presumed enzyme base (step b); molecular oxygen adds to the newly formed anion (step c); and an electronically excited state oxyluciferin molecule and CO_2 are produced from a highly reactive dioxetanone intermediate (steps d and e). According to the original theory (*5, 6*) based predominantly on studies of the *P. pyralis* enzyme, red light emission (λ_{max} 615 nm), which is observed at pH ∼6.0, results from the keto form of the emitter. At pH 7.8, the familiar yellow−green light emission (λ_{max} 560 nm) is produced from the enolate dianion form of the excited state oxyluciferin by a presumed enzymatic assisted tautomerization (step f). In nature, beetle luciferases, which all utilize the same firefly luciferin substrate, display various colors of light from green (λ_{max} ∼530 nm) to red (λ_{max} ∼635 nm) (*7, 8*).

Color modulation has also been attributed to alterations in firefly luciferase structures resulting in changes of polarity (*9*) or rigidity (*10, 11*) of the emitter binding site. In the latter theory (*10, 11*), conformations of the keto form of oxyluciferin, related by rotation about the C2−C2′ bond (Figure 1), account for the color variation. Red emission is attributed to the minimum energy conformation of an unusual twisted intramolecular charge transfer (TICT)[1] excited state (Figure 1) in which the aromatic rings of oxyluciferin are rotated 90° about the C2−C2′ bond. Yellow−green light emission would result from a higher energy excited state conformer. The proposed mechanisms for bioluminescence color have been reviewed (*10*−*12*).

† This work was supported by a grant from the National Science Foundation (MCB 9816898) and the Hans & Ella McCollum-Vahlteich '21 Endowment. Portions of this work were presented at the 10th International Symposium on Bioluminescence and Chemiluminescence, Bologna, Italy, September, 1998.

* To whom correspondence should be addressed at the Department of Chemistry, Connecticut College, 270 Mohegan Ave., New London, CT 06320. Telephone: (860) 439-2479. Fax: (860) 439-2477. E-mail: brbra@conncoll.edu.

[1] Abbreviations: BPTC, 2-(4-benzoylphenyl)thiazole-4-carboxylic acid; CB, 50 mM Tris-HCl (pH 7.0), 150 mM NaCl, 1 mM EDTA, and 1 mM DTT; GST, glutathione-*S*-transferase; PheA, phenylalanine-activating subunit of gramicidin synthetase 1; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; TICT, twisted intramolecular charge transfer; WT, wild-type *Photinus pyralis* luciferase containing the additional N-terminal peptide GPLGS.

10.1021/bi991181o CCC: $18.00
Published on Web 09/14/1999

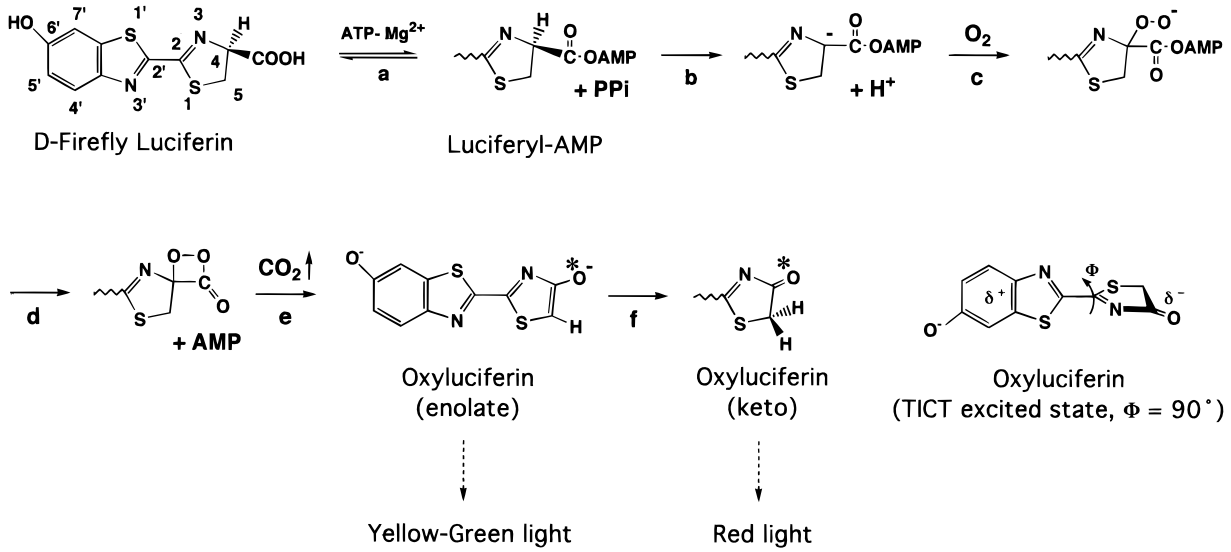

FIGURE 1: Mechanism of firefly luciferase catalyzed bioluminescence and TICT excited state of oxyluciferin.

Mutational studies of firefly luciferases have been conducted (*12*−*18*) that have identified changes in single and/or groups of amino acids widely distributed throughout the sequences of nine luciferases that affect the color of bioluminescence. The comparative studies of these luciferase sequences from five firefly species—*Hotaria parvula* (*15*), *Pyrocoelia miyako* (*15*), *Luciola cruciata* (*14*), *Luciola mingrelica* (*16, 17*), and *P. pyralis* (*13*)—and the four click beetle [*Pyrophorus plagiophthalamus* (*12, 18*)] isozymes have not yielded any direct structure−function relationship to account for the color variation of firefly bioluminescence.

The cloning and sequencing of *P. pyralis* luciferase and similar enzymes from several beetle species (*12, 19*) have revealed that the luciferases are closely related to a large family of nonbioluminescent proteins (*20, 21*) that catalyze reactions of ATP with carboxylate substrates to form acyl adenylates. The *P. pyralis* luciferase crystal structure without bound substrates (*22*), the first one from the "acyl-adenylate/thioester-forming" enzyme family (*23*), revealed a unique molecular architecture consisting of a large N-terminal domain (residues 1−436) and a small C-terminal domain (residues 440−550). A general location of the *P. pyralis* luciferase active site was proposed (*22*) based on an analysis of the positions of conserved residues among approximately 40 enzymes sharing the adenylation function. However, the exact locations of the substrate binding sites could not be established (*22*). Subsequently, the crystal structure of a second member of the adenylate family, the phenylalanine-activating subunit of gramicidin synthetase 1 (PheA) in a complex with Phe, Mg ion, and AMP, was reported (*24*). Starting with the crystal structures of *P. pyralis* luciferase (*22*) and PheA (*24*), we used molecular modeling techniques to produce a potential model of the active site containing substrates luciferin and Mg-ATP (*25*). Recently, the crystal structure of *P. pyralis* luciferase containing two molecules of bromoform, a general anesthetic and luciferease inhibitor, was described (*26*). One of the bromoform molecules binds in a site tentatively identified as the luciferin binding pocket, based on the location of the phenylalanine binding site in the PheA structure (*24, 26*). The newly proposed (*26*) luciferin site binding is in good general agreement with our proposal (*25*).

Previously, we had reported (*27*) that 2-(4-benzoylphenyl)-thiazole-4-carboxylic acid (BPTC) was a potent active site-directed photoinactivation reagent for *P. pyralis* luciferase. The degradation of a luciferase peptide, 244HHGF247, was directly correlated to the photoinactivation process. The results of a subsequent mutagenesis study (*25*) established His245 as the likely primary target of BPTC-catalyzed enzyme inactivation. According to our proposed molecular model (*25*), helix residues 245HGF247 comprise part of the luciferase binding pocket for luciferin. The His245 side-chain imidazole group is likely positioned at the opening of this binding pocket. However, the His245 side chain was not found to be an essential group for productive luciferin binding or efficient light production. Interestingly, all of the His245 mutants catalyzed the emission of light differing from the characteristic yellow−green of native and wild-type (WT) *P. pyralis* luciferase. Since it is very likely that oxyluciferin binds similarly to luciferin, we used our molecular model to determine that, in addition to His245, the side-chain groups of residues Thr343 and Lys529 also might be in close proximity to the thiazolinone ring of the emitter. We report here the results of new mutational studies of His245, Thr343, and Lys529 directed at the mechanistic question of color determination in firefly bioluminescence.

MATERIALS AND METHODS

Materials. *P. pyralis* cDNA in the vector pSx(T7)-Ppy was a generous gift of Monika Gruber. The following items were obtained from the indicated sources: Mg-ATP (equine muscle) (Sigma); luciferin (Biosynth AG); restriction endonucleases, T4 polynucleotide kinase, and T4 DNA ligase (New England Biolabs); and mutagenic oligonucleotides (Genosys). Glutathione-*S*-transferase (GST) fusion constructs, encoding WT and mutant fusion proteins, were expressed and purified as previously described (*25*).

General Methods. Firefly luciferase bioluminescence activity was determined using flash height-based light assays. The standard activity assays were performed in 25 mM glycylglycine buffer (pH 7.8) as described previously (*25, 28*−*30*). All flash height measurements were made by injecting the initiating solutions with a Hamilton Microlab 900 automatic dispenser operated at the maximum speed. The estimated mixing time was ∼0.2 s. Data were acquired

with an SLM-Aminco Chem-Glow II Photometer interfaced to a Strawberry Tree, Inc., A/D converter operated at a sampling rate of 0.05 s. All light measurements were corrected for the spectral response of the Hamamatsu 931B photomultiplier tube used in the assays. The corrections applied were based on the corrected bioluminescence emission spectra obtained as described below. Emission spectra for previously reported *P. pyralis* luciferase mutants (*25*) were remeasured, and specific activity data were recalculated, resulting in several minor changes (Table 1).

The following measurements were performed as described previously: guanidinium chloride-induced denaturation of the luciferases monitored by fluorescence spectroscopy (*25, 31*); circular dichroism spectroscopy (*25*); and steady-state kinetic constants for luciferin and Mg-ATP (*25, 29, 30*).

Site-Directed Mutagenesis. Mutations at H245, K529, and T343 were created by oligonucleotide-directed mutagenesis (*32*) as previously described (*25*). The following mutagenic primers were designed for annealing onto the ssDNA template: H245R, 5′-T TTA AGT GTG GTA CCA TTC CAT **CGC** GGT TTT GG-3′[*Kpn*I]; H245Q, 5′-T TTA AGT GTG GTA CCA TTC CAT **CAG** GGT TTT GG-3′ [*Kpn*I]; H245N, 5′-T TTA AGT GTG GTA CCA TTC CAT **AAC** GGT TTT GG-3′[*Kpn*I]; K529R, 5′-GGT CTT ACC GGT **AGG** CTC GAC GCA A-3′ [*Age*I]; K529Q, 5′-GTA CCG AAA GGG TTA ACC GGA **CAA** CTC GAC GCA-3′ [*Hpa*I]; T343S, 5′-A GGG ATA CGC CAA GGA TAT GGG CTC **TCT** GAG ACT ACA TCA-3′ [*Sty*I]; T343A, 5′-A GGG ATA CGC CAA GGA TAT GGG CTC **GCT** GAG ACT ACA-3′ [*Sty*I] (underline represents silent changes to create a unique screening endonuclease site, boldface represents the mutated codon, and brackets indicate the endonuclease used). The primer 5′-CAC ACA GTA GAC AGG ATC ATG GAA GAC G-3′ (underline represents a new *Acc*I site) was designed to align the WT cDNA reading frame for subcloning (*25*) into the pGex-6p-2 fusion vector and was included in each mutagenesis reaction. The entire cDNA of all luciferase genes was verified by DNA sequencing.

Protein Analysis. Luciferase protein concentrations were determined by UV spectroscopy using $\epsilon_{278} = 45\,560\ M^{-1}\ cm^{-1}$ (*33*). For solutions containing DTT, the Bio-Rad Protein Assay was used with BSA as the standard. SDS−PAGE was performed according to the method of Laemmli (*34*), and gels were stained with Coomassie R-250.

Bioluminescence Emission Spectra. Emission spectra of bioluminescence reactions were obtained over the pH range 5.5−9.0 using a Perkin-Elmer LS-5B luminescence spectrometer operated in the "phosphorescence" mode with the excitation source shunted, the time delay set to 0.1 ms, and the gate time set to 5.5 ms. For bioluminescence measurements, the "phosphorescence" mode is more sensitive than the previously used (*30*) "fluorescence" mode. A pulsed excitation source is activated, and gated detection periods are used to accumulate signal intensity in both modes. In the "fluorescence" mode, however, signals also are sampled during a second gated detection period, which is coordinated to a time period when the excitation source is gated off. The second measurement is subtracted from the first to remove background dark current. When measuring continuously emitting bioluminescence signals in the "fluorescence" mode, the subtraction feature, which cannot be shut off, actually subtracts real signal intensity, thereby lowering sensitivity significantly. The subtraction feature is inactive in the "phosphorescence" mode, and dark current is subtracted prior to the initiation of the bioluminescence reactions.

Reaction mixtures containing WT or mutant luciferases (2−300 μg) in 0.2 mL of CB (0.3 mL of CB for the T343 mutants and a WT control), luciferin, and Mg-ATP (Table 1) were brought to a final volume of 0.5 mL with 25 mM buffer: MES (pH 5.5−6.5); glycylglycine (pH 7.8); or Tris (pH 8.0−9.0). The pH of the mixtures was verified after each emission spectrum was measured. Approximately 2 min after initiation of bioluminescence, spectra were recorded in a 1.0 mL optical glass fluorescence cell at a scan rate of 60 nm/min. Separate measurements of emission intensity for each reaction indicated decreases of no more than ~5% during the recording interval. All emission spectra were corrected for the spectral response of the Perkin-Elmer R928 photomultiplier tube employed, and these spectra were used to determine the spectral corrections for data collected with the light assay device described above.

Molecular Modeling. The starting conformation of oxyluciferin and AMP within *P. pyralis* luciferase was determined by modifying the previously reported lowest energy conformation of luciferase in a complex with luciferin, ATP, and Mg^{2+} (*25*). The low-energy conformations used to draw Figure 6 were generated by conducting Monte Carlo searches on the starting complex. Two searches of 5000 Monte Carlo steps each were conducted as described earlier (*25*) using the following: a "hot" sphere of 12 Å around the oxyluciferin; short restraints between the oxyluciferin phenolate ion and two of the side-chain Arg218 NH's (desired distance 2.5 Å with a half-width flat bottom of 1.5 Å and a force constant of 200 kJ/Å^2); and translation and rotation of the oxyluciferin by between 0 and 1.00 Å and 0 and 180° per step. Conformational families were determined by cluster analysis and by filtering hydrogen bonding interactions in the active site.

RESULTS

Overexpression, Purification, and Characterization of Luciferase Proteins. WT and mutant luciferases listed in Table 1 were expressed as GST-fusion proteins, which, after protease cleavage, contained the additional N-terminal peptide GPLGS. Proteins were purified to homogeneity (as judged by SDS−PAGE) in yields of 8−14 mg/L of culture volume. All enzymes maintained full activity for at least 10 months when stored at 4 °C in CB containing 0.8 M ammonium sulfate and 2% glycerol.

The far-UV circular dichroic (CD) spectra (data not shown) of the His mutants and WT were nearly indistinguishable, indicating that mutations at position 245 had little, if any, effect on secondary structure. The tertiary structure and thermodynamic stability of the H245 mutants appear to be very similar to those of WT, as judged by nearly superimposable fluorescence emission spectra and identical guanidinium chloride-induced denaturation curves with the midpoint denaturant concentration equal to 3.5 ± 0.3 M (data not shown). Similarly, the thermostabilities of the K529 and T343 mutants at 37 °C were within 10% of the value for WT (data not shown).

Bioluminescence Activity of the Luciferases. In an earlier study (*25*) of the role of His245 in firefly bioluminescence, we had prepared and characterized luciferase mutant proteins H244F, H245F, H245A, and H245D. Unlike the H244F mutant, the His245 mutants displayed altered emission

Table 1: Specific Activity and Bioluminescence Emission of Luciferase Enzymes

enzyme	rel sp act.[a]	bioluminescence max (nm)[b] pH 7.8	bioluminescence max (nm)[b] pH 5.5	light emission intensity ratio[c] (green/red)
WT	100.0	558	613	3.1 (0.1)[e]
WT[d]	100.0	556	608	3.2 (0.5)[e]
H244F	18.4	557	612	3.7
H245R	0.05	579	618	0.9
H245F	8.0	595	620	0.6
H245A	26.4	604	620	0.5
H245Q	1.6	606	620	0.4
H245N	24.8	613	617	0.3
H245D	0.23	617	618	0.15
K529R	0.16	560	614	2.8
K529Q	0.06	560	610	2.5
T343S[d]	26.4	560	617	2.7
T343A[d]	1.1	617	621	0.1

[a] Specific activity measurements were made as described previously (*25*). WT values are defined as 100 and are equivalent to 1.18×10^{15} photons s^{-1}/mg in flash height-based assays. Data were corrected for the spectral response of the Hamamatsu 931B photomultiplier tube used in the assays. In the standard activity assays, the final concentrations of Mg-ATP and luciferin were 2 mM and 70 μM, respectively. For mutants with elevated K_m values, substrate concentrations were adjusted: H245F and T343A, 3 mM Mg-ATP and 200 μM luciferin; H245D, 4 mM Mg-ATP and 150 μM luciferin; and K529Q, 3 mM Mg-ATP and 300 μM luciferin. [b] Bioluminescence emission spectra were measured as described under Materials and Methods. [c] Ratio of bioluminescence light emission intensity, 550/620 nm, at pH 7.8. [d] Bioluminescence spectra were measured in higher ionic strength solutions as described under Materials and Methods. [e] Measured at pH 5.5.

spectra compared to WT (Table 1, Figure 2). Our proposed model (*25*) of the *P. pyralis* luciferase active site suggests that only the side-chain groups of His245, Thr343, and Lys529 are within 6 Å of C4 or C5 of luciferin (Figure 1). Moreover, all 3 of these residues are conserved in all of the 17 published luciferase sequences (*12*, *18*, *19*). To evaluate the potential functional role of the three invariant residues in bioluminescence color, we expressed, purified, and characterized the following: T343A and T343S; K529R and K529Q; and three additional His245 mutant proteins (H245R, H245Q, and H245N) (Table 1).

Specific activities (photons s^{-1}/mg) for all luciferases were measured and expressed as flash height-based values (Table 1) that relate the maximum achievable overall reaction rate for all steps shown in Figure 1. These specific activity values are useful for making comparisons of the WT and mutant properties. The specific activities of the H245A, H245N, H244F, H245F, and H245Q mutants were reduced ~4-, ~4-, ~5.5-, ~12.5-, and ~62.5-fold, respectively, compared to WT (Table 1). The introduction of charged residues at position 245 had a greater effect on specific activity as the values for H245R and H245D were reduced ~2000- and ~435-fold, respectively. For the position 529 mutants, the conservative substitution K529R caused a considerable ~625-fold lower specific activity compared to WT; however, the loss of the positively charged side chain was far more deleterious as the K529Q mutant had an ~1650-fold reduced specific activity (Table 1). The conservative substitution of Ser for Thr resulted in an ~4-fold decrease, whereas the removal of the hydroxyl functionality reduced the specific activity of T343A by ~90-fold.

Effects of His245, Thr343, and Lys529 Substitutions on Substrate Binding and Catalytic Constants. To evaluate the effect of substitutions at positions 245, 343, and 529 on the kinetic behavior of *P. pyralis* luciferase, the steady-state kinetic parameters for the WT and mutant enzymes were determined. The H245D and H245F mutants exhibited increases in the K_m for luciferin of ~4- and ~6-fold, respectively, whereas the values for all of the other His245 mutants were approximately the same as the WT value of 15 μM. The K_m value of H245R for Mg-ATP was reduced to about half that of the WT; the other H245 mutants had elevated K_m values ranging from an ~1.5-fold increase for H245A to an ~11.5-fold increase for H245D (Table 2). For the position 529 mutants, the conservative substitution K529R had almost no effect on the luciferin K_m value and resulted in an ~4-fold reduction of the Mg-ATP K_m value. Eliminating the positively charged side chain had substantial effects on the K_m values for both substrates, since the K529Q mutant had ~11-fold and ~5-fold increased values for luciferin and Mg-ATP, respectively (Table 2). The K_m values of the Thr343 mutants for luciferin were increased ~1.5- and ~6.5-fold with the Ser and Ala substitutions, respectively. Both Thr343 mutants had increased K_m values for Mg-ATP. T343S showed an ~2.5-fold increase, while the value for T343A increased ~5.5-fold. For all of the luciferase enzymes except H244F, the effects of the single amino acid changes on k_{cat} were greater than those on K_m (Table 2). The k_{cat} value of the H244F mutant decreased ~7-fold while the K_m values for Mg-ATP and luciferin decreased ~8.5-fold and 3-fold, respectively. All of the mutants are catalytically compromised, and their k_{cat} values are proportional to their respective specific activities (Table 2).

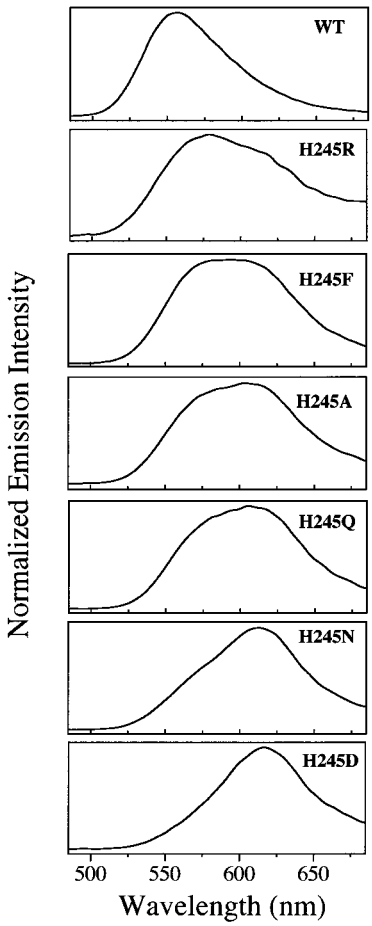

FIGURE 2: Bioluminescence emission spectra of WT and the His245 mutants at pH 7.8. Conditions for recording and correcting the emission spectra are described under Materials and Methods.

Table 2: Steady-State Kinetic Constants for Luciferase Enzymes

enzyme	K_m (μM)[a] luciferin	K_m (μM)[a] Mg-ATP	k_{cat} (s^{-1})[b]
WT	15 ± 2	160 ± 20	1.6 ± 0.3
H244F	5 ± 1	19 ± 2	0.22 ± 0.05
H245R	15 ± 3	95 ± 5	0.00094 ± 0.00023
H245F	89 ± 6	830 ± 30	0.098 ± 0.002
H245A	15 ± 2	240 ± 30	0.52 ± 0.06
H245Q	19 ± 3	380 ± 28	0.027 ± 0.003
H245N	15 ± 2	354 ± 23	0.3 ± 0.02
H245D	64 ± 5	1820 ± 40	0.0037± 0.0003
K529R	16 ± 2	40 ± 10	0.0026 ± 0.0001
K529Q	161 ± 13	829 ± 129	0.0015 ± 0.0001
T343S	27 ± 4	385 ± 69	0.358 ± 0.05
T343A	99 ± 17	857 ± 361	0.0145 ± 0.001

[a] Kinetic constants were determined as described in ref *25*. [b] The k_{cat} values were calculated by dividing the V_{max} values, obtained from the data acquired for the determination of the corresponding K_m values for luciferin, by the concentration of the luciferases present in the assays.

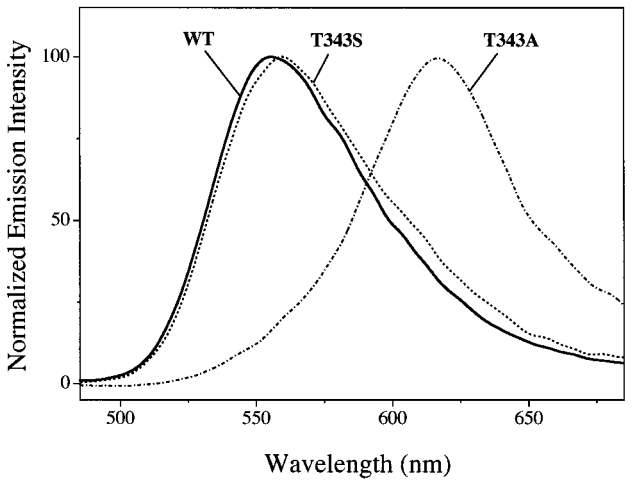

FIGURE 3: Bioluminescence emission spectra of WT and the Thr343 mutants at pH 7.8. Conditions for recording and correcting the emission spectra are described under Materials and Methods.

Bioluminescence Emission. The bioluminescence emission spectra of the luciferases were measured at pH 5.5 and 7.8, and the measured emission maxima are tabulated in Table 1. The corrected and normalized bioluminescence spectra obtained at pH 7.8 for WT and for the His245 and Thr343 mutants are shown in Figures 2 and 3. The colors of light emitted from alkaline and acidic reactions of WT, as well as both of the K529 mutants, H244F and T343S, were almost identical (Table 1 and Figure 3), being green at the higher pH and red at low pH. In contrast, T343A emitted only red light at pH 7.8. The His245 mutants produced broadened emission spectra with increasing maximal emission wavelengths at pH 7.8 from 579 to 613 nm with replacements Arg < Phe < Ala < Gln < Asn (Table 1 and Figure 2). The Asp replacement produced only red light. All of the mutants and WT emit red light at pH 5.5.

DISCUSSION

Roles of His244, His245, Thr343, and Lys529 in P. pyralis Luciferase Bioluminescence. Luciferases with mutations at positions 244 and 529 catalyzed light emission nearly identical in color to WT at both alkaline and acidic pH (Table 1). It is therefore very unlikely that either His244 or Lys529 is involved in bioluminescence color determination. The H244F mutant contains the only substitution found among the luciferases (*12*, *19*) that occurs in the four *Pyrophorus* isozymes (*18*). The H244F mutant had a modestly lower (~5-fold) specific activity; however, the K_m values for both substrates were reduced (Table 2). Compared to WT, the green light emitting click beetle isozyme also has reduced K_m values for luciferin and ATP,[2] possibly as a consequence of having Phe at position 244 (*P. pyralis* numbering).

Lysine at a position equivalent to 529 of WT is absolutely conserved in all luciferases[2,3] (*12*−*18*) and members of the adenylating superfamily (*24*). The importance of a cationic group at position 529 for productive substrate binding is confirmed by the deleterious effects on the K_m values for both luciferin and ATP observed with the K529Q, but not the K529R, mutants (Table 2). Moreover, the k_{cat} values of the Lys529 mutants were greatly reduced (Table 2), indicating a likely functional role for this residue. Additional studies on the role of Lys529 in luciferase are in progress.

In WT, changes at residues His245 and Thr343 affect bioluminescence color. His245 is absolutely conserved in all luciferases, and our model predicts (*25*) that the side-chain imidazole is at the opening of the luciferin binding pocket in close proximity to both the luciferin carboxylate and the γ-phosphate of ATP. The imidazole group appears to be in a position to remove the C-4 proton of luciferin (Figure 1), a critical first step in the enzyme-catalyzed oxidation of the substrate. The k_{cat} value of the H245A enzyme lacking the side chain, however, is reduced only 3-fold compared to WT. This result does not support a major role for the His245 residue in luciferase catalysis. Further, the kinetic properties of the H245A protein (Table 2) suggest that His245 is not necessary for productive luciferin binding and that this residue plays only a minor role in Mg-ATP binding and catalysis of bioluminescence. The steady-state kinetic data (Table 2) for the new His245 mutants reported here (H245R, H245Q, and H245N) support these and earlier conclusions (*25*). Attractive and repulsive ion pair interactions with the γ-phosphate of ATP likely account for the decreased and increased K_m values for Mg-ATP of the H245R and H245D proteins, respectively. Possibly, the considerably lower specific activities of the Arg and Asp mutants also result from ion pair interactions interfering with the release of pyrophosphate during the formation of the luciferyl-adenylate (Figure 1).

Our model of the *P. pyralis* luciferase active site (*25*) predicted that both the main-chain NH and side-chain OH groups of Thr343 form hydrogen bonds to the α-phosphate of ATP. Identical interactions between the equivalent residue (Thr326) and AMP are observed in the crystal structure of PheA (*24*). The T343A mutant lacking the side-chain hydroxyl group binds both enzyme substrates much more poorly than WT and H245A (Table 2). Also, the k_{cat} value of T343A is ~110-fold lower than WT and ~35-fold lower than H245A. Apparently, Thr343 has a more significant role than His245 in both luciferase substrate binding and catalysis. The similar kinetic constants of H245A and the conservative T343S mutant (Table 2) further substantiate the greater relative importance of Thr343. We speculate that in the superfamily of adenylating enzymes, threonine at the position

[2] Monika Gruber, Promega Corp., Madison, WI, personal communication.

[3] V. R. Viviani, personal communication.

equivalent to 343 in *P. pyralis* luciferase participates in orientating ATP for reaction with the carboxylate substrate, and perhaps, in conjunction with Lys529, stabilizes the key pentavalent transition state of the adenylation step.

Firefly Bioluminescence Color: General. Bioluminescence catalyzed by *P. pyralis* luciferase in vivo and at pH 7.8 in vitro is yellow−green with an emission maximum of ∼560 nm. Differences in bioluminescence color are caused by the following: natural species variations in luciferase structure[3] (*7, 8, 12, 18*); amino acid substitutions introduced by mutagenesis techniques (*12*−*18*); in vitro substitutions of analogues of luciferin and ATP for the natural substrates (*3, 5, 30, 35*−*37*); alkylation of cysteine (and possibly other) residues (*38, 39*); and modifications of the in vitro assay conditions, including the addition of certain divalent metal ions (e.g., Hg^{2+} and Zn^{2+}), increasing temperature above ∼25 °C, and maintaining pH in the range ∼5 to ∼7 (*3*). The specific amino acid(s) responsible for the pH sensitivity of the firefly luciferases is (are) not known.

Luciferases[2,3] (*7, 8, 12, 18*) from three families of beetles use identical substrates to generate light ranging in maximum emission wavelength from ∼530 to ∼635 nm. Alterations in luciferase tertiary structure may affect bioluminescence color by causing changes in the polarity of the active site, misalignment of an essential base, thereby disrupting its tautomerization function (Figure 1), or disruption of interactions essential to the maintenance of the proper C2−C2′ dihedral angle of the emitter (Figure 1). It is likely that two distinct excited state emitters produce green light (∼540 nm) and red light (∼630 nm) in beetle bioluminescence. The various colors observed in nature might then be caused by structural variations in the proteins that affect the precise ground state−excited state energy differences of the two emitters. Bioluminescence emission spectra of the fireflies, but not the click beetles and railroad worms, are likely comprised of contributions from both of the emitters, as is apparently the case for WT (Figure 4 and Table 1).

Roles of His245 and Thr343 in Bioluminescence Color. Both His245 and Thr343 are absolutely conserved in the 20 known luciferase sequences[2,3] (*12*−*18*) including 12 from the firefly, 5 click beetles, and 3 "glow worms". Among these sequences, one click beetle isozyme catalyzes orange light emission (*12, 18*), and the railroad worm enzyme produces red light[3]. The bioluminescence emission spectra obtained from the series of His245 mutants reported here as arranged in Figure 2 resemble the broadened red-shifted WT spectra, as the assay pH is adjusted from 7.8 to 5.5 (Figure 4). The protonation of His245 at acidic pH, however, cannot be the direct cause of the red-shifted WT emissions, since all luciferases from the three beetle families have His at position 245 and they are not affected by pH. Moreover, the least altered emission spectrum (λ_{max} = 579 nm) obtained with the His245 mutants was that of H245R which contains the positively charged guanidinium group. Possibly, the imidazolium ion form of His245 may actually favor green light emission.

The H245Q and H245N mutants with side-chain amides isosteric for the Nϵ2 and Nδ1 of His, respectively, were prepared to examine the potential functions of these sites. The emission spectrum produced by H245Q was similar to that of H245A which lacks the imidazole side chain, while the H245N-catalyzed emission closely resembled that of H245D which contains the negatively charged carboxylate

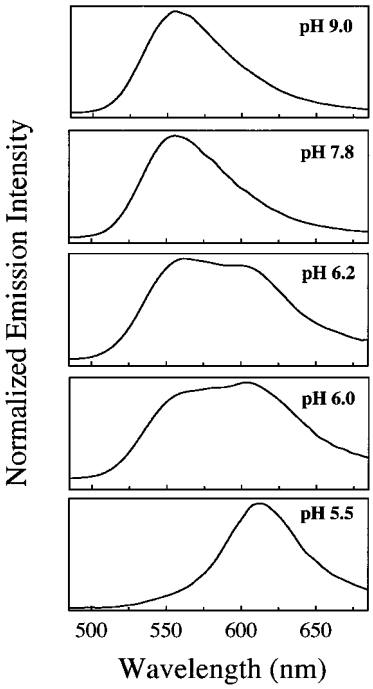

FIGURE 4: Bioluminescence emission spectra of WT over the pH range 5.5−9.0. Conditions for recording and correcting the emission spectra are described under Materials and Methods.

ion (Figure 2). Thus, the hydrogen bonding ability of the Nδ1 and Nϵ2 positions alone is not sufficient for green light emission. The His245 side chain may be necessary to fix the positions of at least two different groups, and this point is discussed further below.

Although the location of His245 in our model (*25*) suggests it might be the luciferase base required in White's (*5, 6*) keto−enol mechanism (Figure 1), this is quite unlikely since the emission spectra of the H245R and H245F mutants, both lacking nucleophilic functional groups, contain significant amounts of green light (Table 1 and Figure 2). Moreover, if the His245 side-chain group were essential, one would expect the bioluminescence of the H245A mutant, lacking any of the steric or functional properties of the His side chain, to be completely red-shifted; however; this was not observed.

His245 is at an N-cap position of a helix that comprises part of the luciferin binding site in our model (*25*). Green light emission appears to be favored by a positive charge at position 245 and/or the maintenance of the helix dipole, as well as by side-chain groups providing substantial nonpolar area (Table 1 and Figure 2). An electropositive region of the emitter site would be expected to interact favorably with the enolate form of oxyluciferin, a feature supporting the theory advanced by White (*5, 6*). However, a properly oriented electropositive center could also fix the keto form of the emitter in a higher energy TICT excited state conformation favoring green light emission according to McCapra's proposal (*10, 11*). The substantially green emission from the H245F mutant, containing the large aromatic side chain, may be a consequence of steric restrictions similarly fixing the conformation of the emitter. The observed color differences among the His245 mutants cannot be accounted for by any single property (*40*) of the amino acids replacing His245. The side-chain imidazole group at position 245, however, appears to be required for normal green light emission in the firefly.

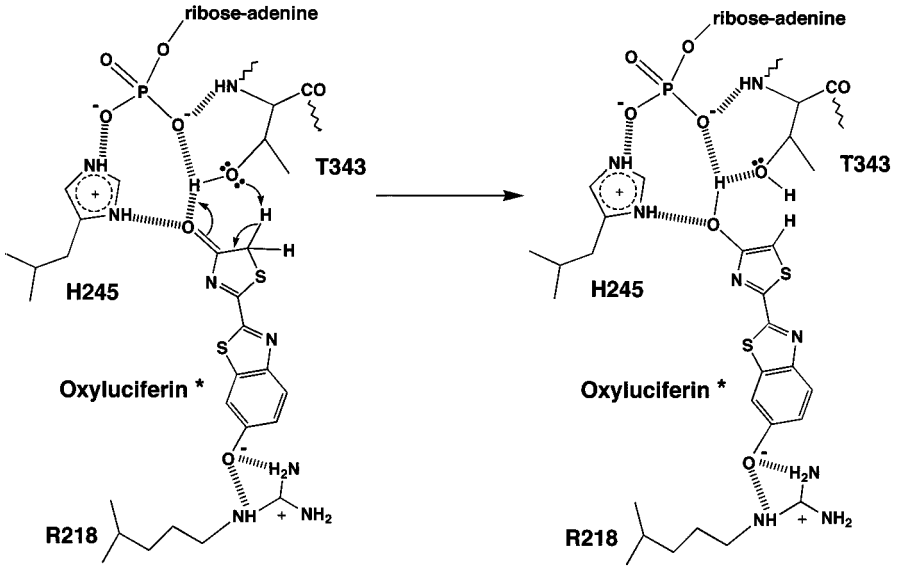

FIGURE 5: Schematic representation of the speculative mechanisms for bioluminescence color determination. The figure on the left indicates the participation of His245, Thr343, AMP, and Arg218 in maintaining a specific conformation of the keto form of excited state oxyluciferin through hydrogen bonding interactions (||||). The tautomerization of the keto form of oxyluciferin to the corresponding enol emitter is indicated by the arrows.

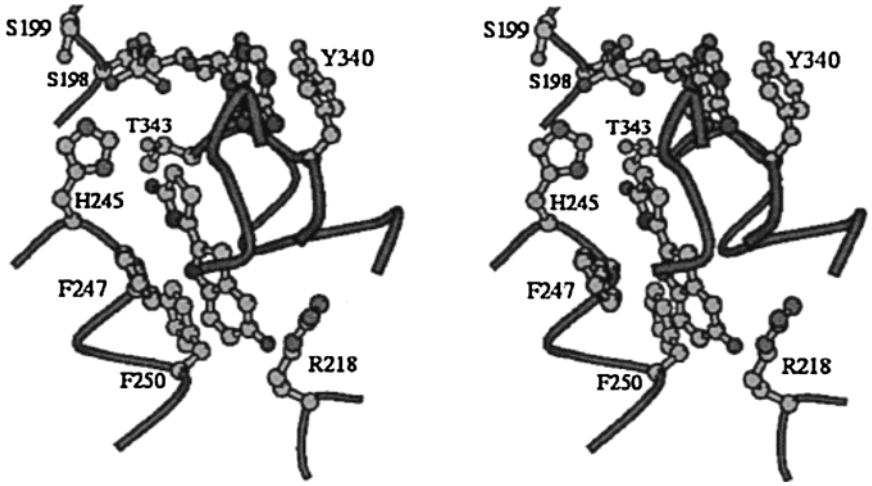

FIGURE 6: Stereo diagram showing the interactions suggested by molecular modeling of *P. pyralis* luciferase with oxyluciferin and AMP. Traces through the α-carbons of regions N197−G200, H244−T252, V217−F219, S314−L319, and Q338−I351 are shown as coils. This diagram was generated using the program MOLSCRIPT (*42*).

The T343S and T343A mutants catalyzed the emission of yellow−green and red light (Figure 3), respectively, and the corresponding emission spectra were nearly identical to those of WT at pH values 7.8 and 5.5 (Figure 4). Apparently, a side-chain hydroxyl group at position 343 is absolutely essential for normal yellow−green light emission in the firefly. In Figure 5, we present a speculative schematic diagram that illustrates how Thr343 may function as an essential determinant of yellow−green light emission. The approximate relative positions of AMP, the keto form of oxyluciferin, and the side chains of His245, Thr343, and R218 shown in Figure 5 are consistent with molecular modeling results (Figure 6) that extend our previous studies (*25*). The side-chain hydroxyl of Thr343 may serve as the required functional group for the abstraction of the luciferin C-5 proton according to White's mechanism (*5*, *6*) as indicated by the arrows in Figure 5. Alternatively, the role of Thr343 (Figure 5) may be to provide an essential hydrogen bond to the keto form of the emitter to maintain a TICT excited state conformer that emits green light as required in McCapra's proposal (*10*, *11*). The participation of His245 and Arg218, residues such as Thr343 that are absolutely conserved among all luciferases, and AMP is indicated in the speculative mechanisms (Figure 5). We propose that Arg218 anchors the benzothiazole ring of the emitter through an ion pair interaction with the phenolate ion (Figures 5 and 6), an interaction identified previously for luciferin and this luciferase residue (*25*). His245 may be required to properly orient both the emitter and Thr343 through hydrogen bonding interactions between the His side-chain group and both the carbonyl of oxyluciferin and a phosphate oxygen of AMP. The hydrogen bonding proton donors could both be from the imidazolium form (Figure 5), or, alternatively, from the neutral form and a bridging water molecule. AMP is included as a key component fixing the position of the Thr343 side chain, enabling it to make an essential hydrogen bond to the emitter carbonyl and possibly removing the C-5 proton. The location of AMP in close proximity to Thr343, His245, and the carbonyl of the emitter is suggested from modeling studies (Figure 6). Based on studies with ATP analogues

(*35*−*37*), the inclusion of AMP is warranted because of the likelihood that it remains bound to luciferase both while the emitter is generated and during the radiative decay process.

Concluding Remarks. The results of mutational and biochemical studies reported here have enabled us to propose speculative mechanisms for color determination in firefly bioluminescence. An essential role for Thr343, the participation of His245 and Arg218, and the involvement of bound AMP are indicated. The proposal can account for the enzyme's role in color determination according to either of the major theories of White and McCapra. Unfortunately, our data cannot be used to distinguish these theories. Although threonine hydroxyl groups (pK ~17) do not commonly function as enzyme bases, our mechanism establishes favorable geometry for a tautomerism process requiring little displacement of the atoms involved. Moreover, the tautomerism of oxyluciferin should be quite facile based on the reported (*41*) extensive enolization of 5-methyloxyluciferin in neutral methanol. We believe that White's mechanism (*5*, *6*) remains viable; however, the simple requirement of tight hydrogen-bonding interactions to maintain normal emission conditions according to the McCapra theory (*10*, *11*) is attractive. Furthermore, we believe that the keto form of oxyluciferin, the red light emitter in both mechanisms, exists in the TICT excited state. In nature, the color variations found among beetles may be modulated mainly by changes in the anisotropic polarity of the emitter site, and residues contributing significantly to this property likely include His245 and Arg218. For the extreme red-emitting luciferases of the click beetle (*12*, *18*) and railroad worm,[3] structural variations in the oxyluciferin binding site may position the emitter so that the functions of Thr343 and/or His245 in producing yellow−green light are no longer possible. Additional mutational studies are currently in progress to better understand the role of specific firefly luciferase residues in the bioluminescence process.

ACKNOWLEDGMENT

We thank Monika Gruber and Justin Stroh for helpful discussions; David Lloyd, John Thompson, and the DNA Sequencing Facility, Pfizer Inc., for providing the DNA sequencing data; Boris Chrunyk for assistance with the circular dichroism studies; and Luke Ward, Jud Grice, and Evelyn Bamford for technical assistance.

REFERENCES

1. Lloyd, J. E. (1978) in *Bioluminescence in Action* (Herring, P. J., Ed.) pp 241−272, Academic Press, New York.
2. Hastings, J. W. (1995) in *Cell Physiology Source Book* (Sperelakis, N., Ed.) pp 665−681, Academic Press, New York.
3. DeLuca, M. (1976) *Adv. Enzymol. 44*, 37−68.
4. Seliger, H. H., and McElroy, W. D. (1960) *Arch. Biochem. Biophys. 88*, 136−141.
5. White, E. H., Rapaport, E., Seliger, H. H., and McElroy, W. D. (1971) *Bioorg. Chem. 1*, 92−122.
6. White, E. H., Steinmetz, M. G., Miano, J. D., Wildes, P. D., and Morland, R. (1980) *J. Am. Chem. Soc. 102*, 3199−3208.
7. McElroy, W. D., and Seliger, H. H. (1966) in *Molecular Architecture in Cell Physiology* (Hayashi, H., and Szent-Gyorgyi, I., Eds.) pp 63−79, Prentice-Hall, Englewood Cliffs, NJ.
8. Hastings, J. W. (1996) *Gene 173*, 5−11.
9. Morton, R. A., Hopkins, T. A., and Seliger, H. H. (1969) *Biochemistry 8*, 1598−1607.
10. McCapra, F., Gilfoyle, D. J., Young, D. W., Church, N. J., and Spencer, P. (1994) in *Bioluminescence and Chemiluminescence: Fundamentals and Applied Aspects* (Campbell, A. K., Kricka, L. J., and Stanley, P. E., Eds.) pp 387−391, John Wiley and Sons, Chichester.
11. McCapra, F. (1997) in *Bioluminescence and Chemiluminescence: Molecular Reporting with Photons* (Hastings, J. W., Kricka, L. J., and Stanley, P. E., Eds.) pp 7−15, John Wiley and Sons, Chichester.
12. Wood, K. V. (1995) *Photochem. Photobiol. 62*, 662−673.
13. Sala-Newby, G. B., and Campbell, A. K. (1991) *Biochem. J. 279*, 727−732.
14. Kajiyama, N., and Nakano, E. (1991) *Protein Eng. 4*, 691−693.
15. Ohmiya, Y., Hirano, T., and Ohashi, M. (1996) *FEBS Lett. 384*, 83−86.
16. Mamaev, S. V., Laikhter, A. L., Arslan, T., and Hecht, S. M. (1996) *J. Am. Chem. Soc. 118*, 7243−7244.
17. Arslan, T., Mamaev, S. V., Mamaeva, N. V., and Hecht, S. M. (1997) *J. Am. Chem. Soc. 119*, 10877−10887.
18. Wood, K. V., Lam, Y. A., Seliger, H. H., and McElroy, W. D. (1989) *Science 244*, 700−702.
19. Ye, L., Buck, L. M., Schaeffer, H. J., and Leach, F. R. (1997) *Biochim. Biophys. Acta 1339*, 39−52.
20. Suzuki, H., Kawarabayasi, Y., Kondo, J., Abe, T., Nishikawa, K., Kimura, S., Hashimoto, T., and Yamamoto, T. (1990) *J. Biol. Chem. 265*, 8681−8685.
21. Babbitt, P. C., Kenyon, G. L., Martin, B. M., Charest, H., Sylvestre, M., Scholten, J. D., Chang, K.-H., Liang, P.-H., and Dunaway-Mariano, D. (1992) *Biochemistry 31*, 5594−5604.
22. Conti, E., Franks, N. P., and Brick, P. (1996) *Structure 4*, 287−298.
23. Chang, K.-H., Xiang, H., and Dunaway-Mariano, D. (1997) *Biochemistry 36*, 15650−15659.
24. Conti, E., Stachelhaus, T., Marahiel, M. A., and Brick, P. (1997) *EMBO J. 16*, 4174−4183.
25. Branchini, B. R., Magyar, R. A., Murtiashaw, M. H., Anderson, S. M., and Zimmer, M. (1998) *Biochemistry 37*, 15311−15319.
26. Franks, N. P., Jenkins, A., Conti, E., Lieb, W. R., and Brick, P. (1998*) Biophys. J. 75*, 2205−2211.
27. Branchini, B. R., Magyar, R. A., Marcantonio, K. M., Newberry, K. J., Stroh, J. G., Hinz, L. K., and Murtiashaw, M. H. (1997) *J. Biol. Chem. 272*, 19359−19364.
28. Branchini, B. R., Magyar, R. A., Murtiashaw, M. H., Magnasco, N., Hinz, L. K., and Stroh, J. G. (1997) *Arch. Biochem. Biophys. 340*, 52−58.
29. Thompson, J. F., Geoghegan, K. F., Lloyd, D. B., Lanzetti, A. J., Magyar, R. A., Anderson, S. M., and Branchini, B. R. (1997) *J. Biol. Chem. 272*, 18766−18771.
30. Branchini, B. R., Hayward M. M., Bamford, S., Brennan P. M., and Lajiness, E. J. (1989) *Photochem. Photobiol. 49*, 689−695.
31. Herbst, R., Schäfer, U., and Seckler, R. (1997) *J. Biol. Chem. 272*, 7099−7105.
32. Zoller, M. J., and Smith, M. (1983) *Methods Enzymol. 100*, 468−500.
33. Green, A. A., and McElroy, W. D. (1956) *Biochim. Biophys. Acta 20*, 170−176.
34. Laemmli, U. K. (1970) *Nature 227*, 680−685.
35. Lee, R., Denburg, J. L., and McElroy, W. D. (1970) *Arch. Biochem. Biophys. 141*, 38−52.
36. DeLuca, M., Leonard, N. J., Gates, B. J., and McElroy, W. D. (1973) *Proc. Natl. Acad. Sci. U.S.A. 70*, 1664−1666.
37. Rosendahl, M. S., Leonard, N. J., and DeLuca, M. (1982) *Photochem. Photobiol. 35*, 857−861.
38. White, E. H., and Branchini, B. R. (1975) *J. Am. Chem. Soc. 97*, 1243−1245.
39. Alter, S. C., and DeLuca, M. (1986) *Biochemistry 25*, 1599−1605.
40. Creighton, T. E. (1993) *Proteins*, 2nd ed., Chapter 4, W. H. Freeman and Company, New York.
41. White, E. H., and Roswell, D. F. (1991) *Photochem. Photobiol. 53*, 131−136.
42. Kraulis, P. J. (1991) *J. Appl. Crystallogr. 24*, 946−950.

BI991181O