Identification of a Lysine Residue at a Nucleotide Binding Site in the Firefly Luciferase with *p*-Fluorosulfonyl[^{14}C]benzoyl-5′-adenosine†

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ABSTRACT: Firefly luciferase is 80–90% inactivated within 3 h upon incubation with the adenine nucleotide analogue *p*-fluorosulfonylbenzoyl-5′-adenosine (FSBA). Although 4 mol of ^{14}C-FSBA/mol of enzyme is irreversibly bound during inactivation, only 1 mol of ^{14}C-FSBA appears to be specifically directed to an adenine nucleotide binding site on the enzyme. The other 3 mol of ^{14}C-FSBA is bound to the protein nonspecifically. The major radioactive peptide in a tryptic digest of labeled luciferase was isolated and shown to have the following amino acid sequence: *Lys-Gly-Glx-Asx-Ser-Lys, where *Lys is the radioactive derivative of the lysine residue that was sulfonylated during the inactivation.

Pal et al. (1975) have reported the synthesis of an adenine nucleotide analogue, *p*-fluorosulfonylbenzoyl-5′-adenosine (FSBA),[1] and have shown it to be an effective reagent for the covalent and irreversible modification of adenine nucleotide binding sites on glutamate dehydrogenase and pyruvate kinase (Pal et al., 1975; Wyatt & Colman, 1977). Esch & Allison (1978b) and Zoller & Taylor (1979) have employed ^{14}C-labeled FSBA in the isolation of covalently modified peptides from the nucleotide binding sites of the mitochondrial ATPase and the skeletal muscle protein kinase.

Firefly luciferase catalyzes the ATP-dependent oxidation of luciferin. The specificity for ATP as a substrate is quite rigorous, as only deoxyATP will substitute for ATP and it is a poor substrate. In this investigation we have used ^{14}C-labeled FSBA to irreversibly inactivate the firefly luciferase as a result of the covalent modification of a lysine residue at or near the nucleotide binding site of the enzyme. The isolation and characterization of the radioactively labeled peptide from tryptic digests of the luciferase are presented here.

Experimental Procedures

Materials. Three times crystallized firefly luciferase was prepared from firefly (photinus pyralis) tails according to the method of Green & McElroy (1956). The enzyme was stored in 10% ammonium sulfate at a concentration of 5–10 mg/mL and was assayed according to the procedure of McElroy & Seliger (1961). Protein concentrations were determined by OD_{280} where 1 mg/mL has an absorbance of 0.75. This was verified by quantitative amino acid analyses. D-Luciferin and dehydroluciferin were synthesized according to Seto et al. (1963). ^{14}C-FSBA with ^{14}C in the benzoyl moiety (Esch & Allison, 1978a) and with a specific radioactivity of 6.8×10^5 cpm/μmol was employed in these studies. The nonradioactive reagent was prepared from adenosine and *p*-fluorosulfonylbenzoyl chloride (Esch & Allison, 1978a) following a modification of the procedure of Pal et al. (1975). N^{ϵ}-(4-Carboxybenzenesulfonyl)lysine (CBS-Lys) and *O*-(4-carboxybenzenesulfonyl)tyrosine (CBS-Tyr) were prepared as previously described (Esch & Allison, 1978b). Trypsin treated with diphenylcarbamoyl chloride was obtained from Calbiochem. Autoradiography was performed with Kodak NS-5T No-Screen medical X-ray film. Purification of peptides via high-pressure liquid chromatography was accomplished with a Waters system consisting of a U6K injector, two Model 6000 solvent delivery systems, and a Model 660 solvent programmer.

Irreversible Covalent Modification of Firefly Luciferase with ^{14}C-FSBA. A solution of 5–10 mg/mL luciferase in 10% ammonium sulfate (typically 30–100 mg) was dialyzed at 4 °C against 2 L of 0.1 M sodium phosphate buffer, pH 8.0, overnight. The enzyme, at a concentration of 5 mg/mL, was 80–90% inactivated within 3 h at room temperature by the addition of nine aliquots of ^{14}C-FSBA given at 20-min intervals where the ratio of ^{14}C-FSBA to enzyme (mole:mole) was approximately 1.5 at each addition. A typical inactivation procedure is described in the legend to Figure 1. Control samples of enzyme incubated under identical conditions except for the additions of ^{14}C-FSBA remained fully active during the 3-h incubations. After 3-h incubation the excess ^{14}C-FSBA was quenched by the addition of solid dithiothreitol to a final concentration of 0.1 M. Five minutes later solid guanidine hydrochloride was added to a final concentration of 5 M and the protein was allowed to denature for 1 h. The radiolabeled, denatured protein was then dialyzed against 2 L of either 1% formic acid or 0.1 M sodium phosphate buffer, pH 8.0 (the latter for spectrophotometric measurements), at 4 °C for 3 days with six changes of dialysate.

Purification of a Radioactive Tryptic Peptide from Luciferase Inactivated with ^{14}C-FSBA. Approximately 100 mg of firefly luciferase was inactivated with ^{14}C-FSBA and treated in a similar manner to that described earlier under Experimental Procedures and in the legend to Figure 1. The modified, denatured, dialyzed, and lyophilized protein was then oxidized with performic acid according to the method of Hirs (1967) and freeze-dried. The labile ester bond linking adenosine to the radioactive carboxybenzenesulfonylated protein was then hydrolyzed (Esch & Allison, 1978b, 1979; Zoller & Taylor, 1979) by incubation of the protein with 0.1 N NaOH for 3–4 h at room temperature. This alkaline hydrolysis permits the isolation of the radiolabeled peptide as a single radioactive species. The NaOH was then removed by gel filtration on a column of Sephadex G-75 (2.7 × 100 cm) equilibrated in 10 mM NH_4HCO_3, and the radioactive fractions were pooled and lyophilized.

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[1] Abbreviations used: ^{14}C-FSBA, *p*-fluorosulfonyl[^{14}C]benzoyl-5′-adenosine; CBS-Lys, N^{ϵ}-(4-carboxybenzenesulfonyl)lysine; CBS-Tyr, *O*-(4-carboxybenzenesulfonyl)tyrosine.

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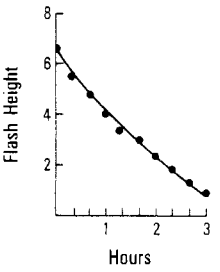

FIGURE 1: Irreversible inactivation of firefly luciferase with [^{14}C]-FSBA. To 30 mg of firefly luciferase in 6 mL of 0.1 M sodium phosphate buffer, pH 8.0, was added 60 μL of 0.0092 M [^{14}C]FSBA in dimethyl sulfoxide every 20 min over a period of 3 h at room temperature. Small aliquots of the incubation mixture were assayed for enzyme activity at 20-min intervals. A control sample containing luciferase was treated with aliquots of 0.0092 M hexamethylphosphoramide in dimethyl sulfoxide at 20-min intervals so that the concentrations of enzyme, hexamethylphosphoramide, and dimethyl sulfoxide were identical with those in the inactivation mixture. This control sample lost no activity during the course of the experiment.

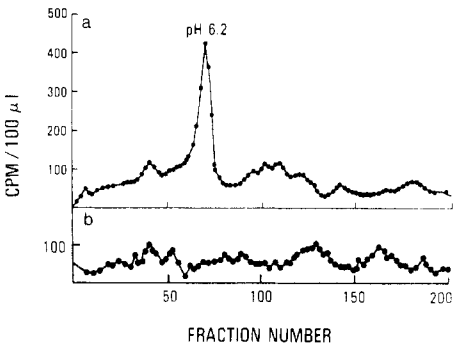

FIGURE 2: (a) Chromatography of a tryptic digest of luciferase labeled with [^{14}C]FSBA on DEAE-Sephadex A-25. The sample was prepared and eluted as described under Experimental Procedures. Aliquots, 100 μL of the 6-mL fractions, were analyzed for radioactivity by liquid scintillation counting. (b) Conditions are the same as in (a) except a 50-fold molar excess of Mg^{2+}ATP was present during the reaction with [^{14}C]FSBA.

The alkaline hydrolysate of the radiolabeled protein was dissolved in a minimal volume of 0.1 M NH_4HCO_3 buffer, pH 8.0, and digested with trypsin at 37 °C for 5 h by using a 1:20 (w/w) ratio of protease to substrate. The digestion was terminated by lyophilization, and the freeze-dried material was subsequently dissolved in 1% pyridine–1% collidine adjusted to pH 7.0 with acetic acid and applied to a DEAE-A25 Sephadex column (2.5 × 5 cm) equilibrated in the same buffer. The column was eluted at 2 mL/min with a pH gradient employing a mixing chamber that contained 500 mL of the initial buffer at pH 7.00 and a reservoir containing 500 mL of 1% pyridine–1% collidine adjusted to pH 5.5 with acetic acid. The elution profile of this column is shown in Figure 2a. The fractions in the major radioactive peak eluting from this column were pooled and lyophilized. The radiolabeled peptide in this material was then purified to homogeneity on a Waters Associates high-pressure liquid chromatograph using a Waters C_{18} μBondapak (7.5 × 300 mm) semipreparative high-performance liquid chromatograph column. The peptide was eluted isocratically with 0.01 M sodium phosphate buffer, pH 4.80, at a flow rate of 3 mL/min as shown in Figure 3. A Schoeffel variable wavelength detector was employed to monitor the column eluant at 210 nm for the presence of peptides. The UV-absorbing peaks in the eluant were collected by hand in separate tubes and assayed for radioactivity by liquid scintillation counting.

Amino Acid Analysis and Peptide Sequence Determination. Protein and peptide samples were routinely hydrolyzed with

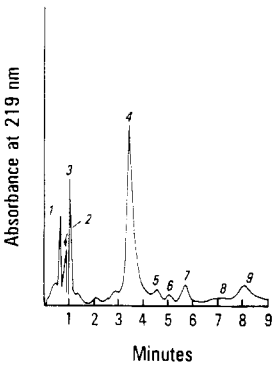

FIGURE 3: High-pressure liquid chromatography purification of the ^{14}C-labeled tryptic peptide. The sample was prepared and eluted as described under Experimental Procedures. Radioactive scintillation counting of aliquots from the eluate revealed that the only radioactive UV-absorbing peak in the chromatograph was peak 4.

6 N HCl at 110 °C for 24 h in sealed evacuated tubes prior to amino acid analyses on a Beckman 119 automatic amino acid analyzer. The amino acids were eluted from a 150 × 0.9 cm. column filled with Beckman AA-15 ion exchange resin with sodium citrate buffers having pH values of 3.27, 4.12, and 6.40 (Beckman, 1974). The sequence of the isolated radiolabeled peptide was determined by using a classical dansyl chloride/Edman degradation technique (Gray & Smith, 1970) with the dansylated amino acids being identified by thin layer chromatography (Morse & Horecker, 1966). The location of the ^{14}C-labeled amino acid was ascertained by the relative release of radioactivity at each cycle of the Edman degradation.

Results and Discussion

Stoichiometry of ^{14}C Incorporation into Luciferase after Inactivation with ^{14}C-FSBA. The extent of incorporation of ^{14}C-FSBA into the enzyme after inactivation with the reagent was determined by two independent methods. With the assumption that the extinction coefficient of FSBA (ϵ = 15.8 mM^{-1} at 259 nm) (Pal et al., 1975) is not appreciably altered upon reaction with luciferase, it was determined from spectrophotometric analyses of the native and modified enzymes that approximately 4 mol of FSBA were incorporated per mole of enzyme. Radioactive scintillation counting of small portions of the ^{14}C-FSBA-modified enzyme confirmed that 4 mol of ^{14}C-FSBA was covalently bound to the denatured, exhaustively dialyzed protein.

It has been shown that the presence of ATP–Mg^{2+} and dehydroluciferin, a substrate analogue, will protect the luciferase from inactivation by sulfhydryl reagents (DeLuca et al., 1964). The presence of these substrates also induces a large conformational change in the enzyme (DeLuca & Marsh, 1967). We therefore examined the effect of ATP–Mg^{2+} and dehydroluciferin on the inactivation of the luciferase with FSBA. Two samples of enzyme were incubated with ^{14}C-FSBA as described in the legend to Figure 1 except that one sample contained a 50-fold excess of ATP–Mg^{2+} and the other sample contained a 10-fold excess of dehydroluciferin and ATP–Mg^{2+} over the enzyme. A control sample containing no substrates was also run. After 3-h incubation at room temperature, no significant inhibition (0 to 5%) of enzyme activity by ^{14}C-FSBA was observed in the samples with substrates. These results suggest that ATP–Mg^{2+} can block inhibition of the enzymatic activity by ^{14}C-FSBA. The incubation mixture was then treated with dithiothreitol and guanidine–HCl and dialyzed against 1% formic acid as described under Experimental Procedures; 2.2–2.7 mol of ^{14}C-FSBA/mol of enzyme was found covalently bound to the

Table I: Amino Acid Composition of the ^{14}C-Labeled Tryptic Peptide from Luciferase Inactivated with [^{14}C]FSBA

amino acid	no. of residues[a]	amino acid	no. of residues[a]
Asp	1.23	Gly	0.99
Ser	0.77	Cbs-Lys	1.00[b]
Glu	0.99	Lys	1.00

[a] Relative to lysine. [b] Amount of Cbs-Lys was estimated by amount of ^{14}C present in the hydrolysate.

denatured, exhaustively dialyzed enzyme by liquid scintillation counting and protein concentration determination procedures. It appears that the reaction of 1 mol of ^{14}C-FSBA/mol of enzyme is associated with the loss in enzymatic activity. The remaining 2–3 mol of ^{14}C-FSBA which are bound per mole of enzyme are due to nonspecific labeling as discussed later.

Identification of the Amino Acid Residue Labeled with ^{14}C-FSBA. The side chains of serine, tyrosine, lysine, and histidine residues in proteins are capable of reacting with sulfonyl halides (Paulos & Price, 1974). Since sulfonylated serine and histidine are labile (Paulos & Price, 1974), only the sulfonylated derivatives of tyrosine and lysine were synthesized as standards for the identification of the amino acid residue labeled by ^{14}C-FSBA in luciferase.

Approximately 30 nmol of the ^{14}C-FSBA-labeled enzyme was hydrolyzed with 6 N HCl at 110 °C for 21 h. The acid hydrolysates were subjected to paper electrophoresis at pH 8.9 for 1.5 h at 1.5 kV along with samples of CBS-Lys and CBS-Tyr which had been treated under similar conditions. The carboxybenzenesulfonylated amino acid derivatives and the derivative found in the hydrolysate of the ^{14}C-labeled enzyme could be detected with an ultraviolet lamp. The UV-absorbing spots were circled, and the chromatogram was radioautographed for 24 h and finally stained with fluorescamine (Mendez & Lai, 1975). The combination of fluorescamine staining, which detects lysine, tyrosine, CBS-Lys, and CBS-Tyr, and radioautography, which detects the ^{14}C-labeled amino acid in the protein hydrolysate, confirmed the identification initially made with the ultraviolet lamp. ^{14}C-CBS-Lys was unequivocally identified as the labeled amino acid in the protein hydrolysate.

This result was further substantiated by comparing amino acid analyses of the homogeneous ^{14}C-labeled tryptic peptide and synthetic CBS-Lys and CBS-Tyr samples. CBS-Lys elutes as a very distinctive ninhydrin-positive component between norleucine and tyrosine when a standard Beckman elution program (Beckman, 1974) is used. This peak was readily detected in acid hydrolysates of the purified ^{14}C-labeled tryptic peptide.

Isolation, Amino Acid Analysis, and Sequence Determination of the Tryptic Peptide Containing ^{14}C-CBS-Lys. The major radioactive peptide in a tryptic digest of the ^{14}C-FSBA-inactivated luciferase was purified to homogeneity by alkaline hydrolysis of the labile ester bond in ^{14}C-FSBA, gel filtration, ion-exchange chromatography and high-pressure liquid chromatography as described in detail under Experimental Procedures. The elution of a high background of radioactivity during the ion exchange chromatography purification step shown in Figure 2a and the recovery of only 25% of the total radioactivity loaded onto that column in the major radioactive peak (which elutes at approximately pH 6.2) suggests that a large amount of the ^{14}C-FSBA incorporated into the luciferase during inactivation is nonspecific. This hypothesis is strongly supported by the results obtained by using an identical ion-exchange chromatography procedure on a tryptic digest of the ^{14}C-FSBA-labeled lucifierase which had been modified in the presence of ATP or dehydroluciferin and ATP. The fully active enzyme had incorporated 2.2–2.7 mol of ^{14}C-FSBA/mol of enzyme, and the radioactive elution profile from the ion-exchange column run on the tryptic digest showed a high background of radioactivity indicative of nonspecific labeling, with the conspicuous absence of a major radioactive species eluting at approximately pH 6.2 (Figure 2b). While 4 mol of ^{14}C-FSBA/mol of enzyme is incorporated into the luciferase during the inactivation of the enzyme, it appears likely that only 1 mol of ^{14}C-FSBA reacts at or near the ATP binding site. The other 3 mol of ^{14}C-FSBA is apparently incorporated into the protein nonspecifically and results in the high radioactive background of the elution profile observed during ion-exchange chromatography of the labeled tryptic digest.

Quantitative amino acid analysis, presented in Table I, showed that the major isolated ^{14}C-labeled tryptic peptide contained only six residues. Approximately 300 nmol of the homogeneous peptide was subjected to a manual Edman degradation with the amino-terminal amino acid at each cycle identified by dansylation and thin-layer chromatography. The amino acid sequence of this peptide was determined to be ^{14}C-CBS-Lys-Gly-Glx-Asx-Ser-Lys. The relase of 79% of the peptide's total radioactivity in the ethyl acetate layer of the first cycle (Gray & Smith, 1970) identified the first residue as ^{14}C-CBS-Lys.

It is interesting that this peptide, presumably derived from the ATP binding site, has no apparent similarity with the active-site sulfhydryl peptide isolated and sequenced by Travis & McElroy (1966). It is also completely different from a peptide obtained from the luciferin binding site reported by Lee & McElroy (1971). The fact that only one FSBA was required to inactivate the enzyme is noteworthy. This confirms a previous observation that the luciferase has one binding site for Mg^{2+}–ATP per 100 000 daltons of enzyme (Lee et al., 1970). Two moles of ATP will bind in the absence of Mg^{2+}, and one might have expected to find another site specifically labeled with FSBA. Apparently the FSBA bound only to the Mg^{2+}–ATP site.

Acknowledgments

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Cobalt Tyrosinase: Replacement of the Binuclear Copper of *Neurospora* Tyrosinase by Cobalt†

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ABSTRACT: The antiferromagnetically spin-coupled copper(II) pair in *Neurospora* tyrosinase was substituted by cobalt, yielding a stoichiometry of 2 mol of Co/mol of protein. The low magnitude of the high-spin Co(II) EPR signal indicates spin coupling of the two Co(II) ions similar to that observed in the native enzyme. The absorption spectrum with four transitions in the visible region of intermediate intensity (ϵ_{607} 670, ϵ_{564} 630, ϵ_{526} 465), a shoulder at 635 nm, and the near-infrared bands at 1180 (ϵ 30) and 960 nm (ϵ 15) indicate tetrahedral coordination around the Co(II) center. The cobalt(II) tyrosinase is enzymatically inactive, and there is no evidence that it binds molecular oxygen. Upon addition of cyanide or the competitive tyrosinase inhibitors L-mimosine, benzoic acid, or benzhydroxamic acid the absorption spectrum changes in a characteristic manner. This optical perturbation shows that binding of these inhibitors (and presumably of the substrates) occurs at or near the metal site. One Co(II) ion can be removed preferentially by incubation with KCN at high pH, indicating the two ions not to be in an identical environment.

Tyrosinase (EC 1.14.18.1) is a copper-containing monooxygenase which catalyzes the oxidation of monophenols to *o*-diphenols (cresolase activity) and of *o*-diphenols to *o*-quinones (catecholase activity) (Mason, 1965). The enzyme was purified from different microorganisms, plants, and animals, and it is involved in the formation of melanins and other polyphenolic compounds (Vanneste & Zuberbühler, 1974; Nicolaus, 1968). *Neurospora* tyrosinase is a single-chain protein with a molecular weight of 46 000 containing 2 mol of copper/mol of protein (Lerch, 1976, 1978). The copper was found to be EPR[1] nondetectable, attributable to an antiferromagnetically spin-coupled copper pair (Deinum et al., 1976) as was demonstrated earlier for mushroom tyrosinase from *Agaricus bispora* (Schoot Uiterkamp & Mason, 1973). Resonance Raman studies showed that the oxygen in *Neurospora* oxytyrosinase is bound to the protein as peroxide (Eickman et al., 1978), as was previously reported for oxyhemocyanin (Freedman et al., 1976). In addition, these two proteins share other properties: both contain type 3 copper (Fee, 1975), display virtually the same absorption spectra, and have similar EPR-sensitive NO- and half-met derivatives (Schoot Uiterkamp & Mason, 1973; Eickman et al., 1979; Himmelwright et al., 1980). The two proteins, however, fulfill quite different functions (monooxygenase vs. oxygen transporting function). This must be reflected in their active centers which in the case of tyrosinase must contain a substrate binding site (lacking in hemocyanin) in addition to the oxygen binding site. From photooxidation (Pfiffner et al., 1980) and active-site-directed modification experiments (Dietler & Lerch, 1979) in conjunction with amino acid sequence data of *Neurospora* tyrosinase (Lerch, 1978), it was suggested that histidyl residues 188, 193, 289, and 306 represent possible ligands to the copper site in this enzyme. For more information on the active site of this monooxygenase, the copper of the native enzyme was chemically replaced by cobalt. Complexes of cobalt(II) exhibit characteristic d–d transitions which are dependent on the ligand geometry and which are very sensitive to changes in the immediate environment of the metal. Thus Co(II) substitution can be used to study the overall geometry and the interaction of ligands with the metal site in metalloproteins. Cobalt(II) derivatives have been prepared of carboxypeptidase A, carbonic anhydrase, alkaline phosphatase (Vallee & Wacker, 1970), phosphoglucomutase (Ray et al., 1972), hemoglobin (Hoffman & Petering, 1970), horseradish peroxidase (Wang & Hoffman, 1977), liver alcohol dehydrogenase (Sytkowski & Vallee, 1976), superoxide dismutase (Calabrese et al., 1972), stellacyanin (McMillin et al., 1974), rubredoxin (May & Kuo, 1978), and *Rhus* laccase type 1 (Larrabee & Spiro, 1979). In these Co derivatives cobalt forms a mononuclear complex with the protein moiety. Tyrosinase, however, contains a binuclear copper site, and hence a binuclear cobalt site is expected in cobalt(II) tyrosinase. Substitution of the oxygen-binding copper atoms of native tyrosinase with cobalt is also of special interest since a large number of low molecular weight Co(II) complexes are known to bind oxygen reversibly as a binuclear complex (Wilkins, 1971). In contrast, information on copper model complexes fulfilling a similar function is still severely limited.

This report describes the preparation and some properties of cobalt(II)-substituted tyrosinase from *Neurospora crassa*. The relationship of cobalt(II) tyrosinase to the binuclear cobalt model complexes as well as to the mononuclear cobalt substituted proteins is discussed.

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[1] Abbreviations used: EPR, electron paramagnetic resonance; CD, circular dichroism.

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