Isolation and Sequence of an Essential Sulfhydryl Peptide at the Active Site of Firefly Luciferase*

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ABSTRACT: The reaction of the competitive inhibitor dehydroluciferin with adenosine triphosphate (ATP) in the presence of firefly luciferase has been reported to result in the masking of two of the six sulfhydryl groups of the enzyme [DeLuca, M., Wirtz, G. W., and McElroy, W. D. (1964), *Biochemistry 3*, 935]. Because the titration of all of the sulfhydryl groups in luciferase results in total inhibition it was of interest to determine the amino acid sequence in the vicinity of the protected sulfhydryl groups. Labeling of the essential sulfhydryl groups with [1-^{14}C]*N*-ethylmaleimide followed by tryptic digestion resulted in the isolation of a single radioactive decapeptide whose sequence was determined.

In view of these results molecular weight studies of native and denatured luciferase together with amino-terminal analyses and tryptic peptide patterns of the enzyme were undertaken. The results indicate that firefly luciferase is composed of two like monomeric units of mol wt 50,000.

Firefly luciferase is known to catalyze both the formation and oxidation of enzyme-bound luciferyl adenylate[1] resulting in the production of light. In addition, luciferase will also bring about the formation of the tightly bound competitive inhibitor, dehydroluciferyl adenylate. Although a study of these reactions has already been reported (McElroy and Seliger, 1961; Rhodes and McElroy, 1958) little is known about the physical and chemical structure of the enzyme.

The crystallization and purification of firefly luciferase was first described by Green and McElroy (1956). The enzyme has a molecular weight near 100,000 and an isoelectric point between pH 6.2 and 6.3 It contains six to seven free SH groups/mole which react with PMB to yield a completely inhibited product (DeLuca *et al.*, 1964). However, in the presence of dehydroluciferyl adenylate two of these SH groups are specifically protected from PMB reaction. Furthermore, subsequent removal of dehydroluciferyl adenylate from the titrated enzyme results in the recovery of >90% of the original enzymatic activity. As a result of the above studies it became of interest to us to determine the amino acid sequence in the vicinity of these two SH groups by alkylation with [1-^{14}C]NEM.

We wish to report here both the isolation and amino acid sequence of a cysteinyl-containing decapeptide from tryptic digests of the labeled protein. In addition, evidence is presented which suggests that the native enzyme is actually composed of two like monomeric units.

Materials

Three times crystallized firefly luciferase was prepared by the method of Green and McElroy (1956) and stored at 4° in 10% ammonium sulfate, 10^{-3} M EDTA, pH 7.8, at a concentration of about 10 mg of protein/ml. The enzyme was assayed by the method of McElroy and Seliger (1961) as described by DeLuca *et al.* (1964).

Trypsin was a twice-crystallized product of Worthington Biochemicals. Subtilisin was purchased from the Sigma Chemical Co. DFP-treated carboxypeptidase A was obtained from Worthington Biochemicals and had been recrystallized three times.

Luciferin and dehydroluciferin were prepared according to White *et al.* (1961) and the concentration of each was determined by measuring the absorbance at neutral pH at 327 mμ for luciferin and 347 mμ for dehydroluciferin. DTNB was a product of the Aldrich Chemical Co. and was dissolved in 0.1 M phosphate, pH 7.5, prior to use. Titrations were followed by measuring the absorbance at 412 mμ as described by Ellman (1959). Total SH content was calculated assuming an ϵ_m of 13,600 for the reaction product. [1-^{14}C]NEM was obtained from Schwarz Biochemicals and had a specific activity of 2–4 μc/mg. [^{3}H]FDNB was a product of the New England Nuclear Corp. and was diluted with FDNB prior to use.

Urea and guanidine hydrochloride were both recrystallized prior to use. *N*-Ethylmorpholine (Eastman Organic Chemicals), practical grade, was redistilled (bp 137–139°) and stored at 4° over NaOH pellets. All

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[1] Abbreviations used in this paper: PMB, *p*-mercuribenzoate; NEM, *N*-ethylmaleimide; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); FDNB, 2,4-dinitrofluorobenzene; SSC, *S*-succinylcysteine; E-LH$_2$-AMP, enzyme-bound luciferyl adenylate; E-L-AMP, dehydroluciferyl adenylate; ATP, adenosine triphosphate; NADH, reduced nicotinamide-adenine dinucleotide.

other chemicals were of reagent grade and were used as obtained.

Methods

Preparation of [1-^{14}C]NEM Luciferase. In a typical experiment, 1 μmole of luciferase (90 mg) was allowed to react with a 10:1 molar excess of both dehydroluciferin and ATP for 15 min in 0.05 M Tris buffer, pH 7.1, containing 0.1 M $MgSO_4$, at 4°. DTNB (50 μmoles) was then added and the reaction followed to completion. After 2 hr the reaction appeared to be over and the solution was dialyzed *vs.* repeated changes of the Tris buffer at 4° for 36–48 hr.

At this point 2.5 μmoles of [1-^{14}C]NEM was added, followed immediately by enough solid urea to bring the final concentration to about 6 M. This concentration of urea is sufficient to release all of the dehydroluciferyl adenylate from the enzyme. The reaction mixture was allowed to warm to room temperature at pH 7.1 and, after 3 hr, dialysis *vs.* 10^{-3} M HCl was begun to effect removal of the urea. The protein remained soluble throughout the dialysis and was finally precipitated by the addition of 200 ml of 95% ethanol. It was then washed with acetone and air dried.

Isolation of [1-^{14}C]NEM Peptides. Tryptic digestion of [1-^{14}C]NEM-labeled luciferase was carried out in 0.1 M triethylamine acetate buffer, pH 8.1. Digestions were performed at enzyme–substrate molar ratios of 1:10 for 5 hr at 37°. At the end of this period trypsin was inactivated by lowering the pH to about 3 by the addition of formic acid. No insoluble core was observed and the entire solution was lyophilized.

The dry product was dissolved in 0.05 M acetic acid and applied to a 1.5 × 90 cm column of G-25 Sephadex previously equilibrated against the same solvent. Flow rates were adjusted to 15–18 ml/hr and 2-ml fractions collected. Aliquots of each fraction were analyzed by measurement of both absorbance at 278 mμ and radioactivity. The contents of the tubes containing radioactive material were pooled and lyophilized.

Radioactive fractions obtained from the Sephadex columns were found to contain mixtures of peptides. These were resolved by high voltage paper electrophoresis at pH 1.9, 3.7, and 6.5 using volatile buffer systems as described by Katz *et al.* (1959). Peptides were then visualized by dipping in ninhydrin reagent as prepared by Canfield and Anfinsen (1963). For preparative work, marker strips were stained and the zones corresponding to ninhydrin-positive, radioactive areas were cut out and eluted with 5% acetic acid.

Amino acid analyses of proteins or peptides were determined on acid hydrolysates prepared in 6 N HCl in evacuated, sealed tubes. Samples which were radioactive were hydrolyzed for 72 hr. All other material was digested for 22 hr. The hydrolysates were concentrated to dryness several times in an Evapo-Mix (Buchler Instrument Co.) and analyzed in a Spinco Model 120B amino acid analyzer according to the method of Spackman *et al.* (1958).

SSC, the acid hydrolysis product of the reaction between NEM and the SH groups of proteins, could be determined only after 72 hr of hydrolysis (Smyth *et al.*, 1964). This amino acid derivative eluted at about 65 ml on the 120B analyzer, or just prior to *S*-carboxymethylcysteine.

Sequential Analysis of Peptides. Amino acid sequences were performed by a combination of Edman degradations and enzymic digestion of peptides. The technique of Konigsberg and Hill (1962) was utilized for the Edman reaction with one modification. After cyclization of the phenylthiocarbamyl peptide with trifluoroacetic acid, the solution was taken to dryness and redissolved in 0.2 M acetic acid. It was then passed through a 1 × 10 cm column of G-10 Sephadex equilibrated *vs.* the same solvent. This facilitated a cleaner separation of the residual peptide from other low molecular weight contaminants and allowed the completion of several degradations without the additional purification of peptide residues by ion-exchange chromatography or paper electrophoresis.

Subtilisin digestion of radioactive peptides was performed in 0.1 M triethylamine acetate buffer, pH 8.0, for 12 hr at room temperature at an enzyme–substrate molar ratio of 1:25. Separation of the digest was then effected by paper electrophoresis at pH 3.7 for 90 min at 2000 v.

Carboxypeptidase A digestion of peptides was carried out in 0.1 M ammonium acetate, pH 8.0, for 12 hr at an enzyme–substrate molar ratio of 1:50. Amino acid analysis of the digests was performed without prior removal of the exopeptidase.

Molecular Weight Determinations. Sedimentation velocity and sedimentation equilibrium experiments were carried out in a Beckman Spinco Model E ultracentrifuge. All velocity studies were performed at 5° at a rotor speed of 59,780 rpm. Equilibrium studies were made at a similar rotor temperature except for studies in guanidine hydrochloride which were performed at room temperature.

Two different methods were used for the determinations of molecular weight. Low-speed equilibrium measurements were performed as outlined by Young *et al.* (1964) on solutions of luciferase equilibrated *vs.* 10% ammonium sulfate, 10^{-3} M EDTA, pH 7.8, at concentrations of 5–10 mg of protein per ml. Rotor speeds were between 5220 and 9400 rpm.

Molecular weights were also estimated using the high-speed equilibrium procedure of Yphantis (1964). Solutions of luciferase at concentrations of about 0.15 mg of protein/ml were pretreated with a 70-fold molar excess of NEM to eliminate oxidation of free SH groups. Aliquots were then exhaustively dialyzed *vs.* either 0.1 M Tris buffer, pH 7.0, containing 0.2 M NaCl or unbuffered 5 M guanidine hydrochloride, pH 7.0. Rotor speeds of 21,740 and 37,520 were used for the native and denatured solutions, respectively.

The partial specific volumes of luciferase were taken to be 0.73 ml/g in buffer and 0.71 ml/g in guanidine hydrochloride. The partial specific volume of many proteins decreases about 0.02 ml/g upon denaturation

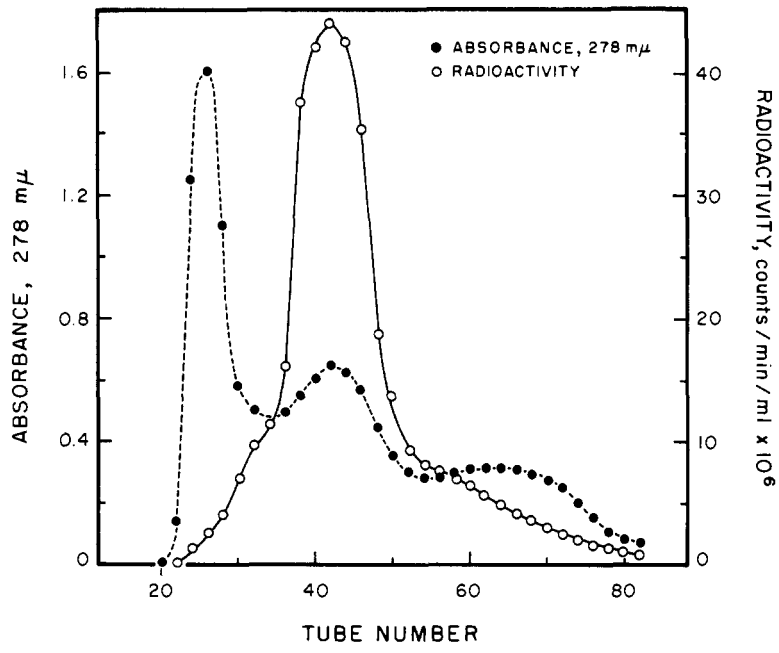

FIGURE 1: Chromatography of tryptic digest of [1-^{14}C]NEM-labeled luciferase on G-25 Sephadex. Elution was performed with 0.05 M acetic acid; ●, absorbance at 278 mμ; ○, radioactivity.

in guanidine hydrochloride (Kielley and Harrington, 1960).

Amino-Terminal Analysis. N-Terminal analyses were performed on 10-mg samples of luciferase with FDNB as described by Fraenkel-Conrat *et al.* (1955). [^{3}H] FDNB was used only for identification of the N-terminal amino acids. Positive identification of the dinitrophenyl (DNP) amino acids was attempted by hydrolysis with $Ba(OH)_2$ (Mills, 1952), followed by amino acid analysis.

Peptide Mapping. Oxidized luciferase (0.1 μmole) was prepared from salt-free samples of native enzyme by the method of Moore (1963) and the oxidized protein digested with trypsin (0.005 μmole) at pH 8.0 for 3 hr at 37°. Fingerprints were prepared as described by Katz *et al.* (1959) with electrophoresis at pH 3.7 and chromatography in *n*-butyl alcohol–acetic acid–water (4:1:5). Staining of the patterns was performed as described above.

Results

Isolation and Composition of Radioactive Peptides. In the presence of dehydroluciferyl adenylate titrations of luciferase with DTNB resulted in the reaction of four of the six SH groups in the enzyme. Amino acid analysis and planchet counting of the isotopically labeled protein verified the titration studies, 1.80 moles of SSC and 1.95 moles of ^{14}C being found/mole of enzyme.

The use of Sephadex for the preliminary separation of peptides is well known. Chromatography of tryptic digests of labeled luciferase on G-25 Sephadex as shown in Figure 1 resulted not only in the removal of trypsin and undigested protein but also in the separation of peptides according to size and aromatic content. The first peak that emerged from the column represented a mixture of trypsin, alkylated luciferase, and large polypeptides. The amount of undigested protein was quite small, however, since over 95% of the radioactivity applied to the column was eluted in the second peak. This material was shown to be composed of a number of ninhydrin-positive peptides after electrophoresis. However, only one was radioactive and after isolation by preparative electrophoresis it was given the designation LT-1.

Although two of the six SH groups in luciferase have already been implicated in enzymatic activity it was decided to test the rate of reactivity of all of the SH groups of the native enzyme by the stoichiometric addi-

TABLE I: Amino Acid Composition of LT-1 and LT-2 Peptides.

Amino Acid Residues/Mole	Peptide	
	LT-1	LT-2
SSC	1.10 (1)[a]	1.05 (1)[a]
Aspartic acid	0.80 (1)	1.10 (1)
Serine	1.95 (2)	2.25 (2)
Glutamic acid	1.62 (2)	2.24 (2)
Glycine	2.30 (2)	1.95 (2)
Alanine	1.02 (1)	1.20 (1)
Lysine	1.01 (1)	0.85 (1)
Total	(10)	(10)

[a] To nearest integer.

tion of NEM. The results of these studies are shown in Figure 2. It may be seen that a loss of >80% of the enzymatic activity can be correlated with the alkylation of two SH groups. Amino acid analysis confirmed these results, 1.75 moles of SSC being formed/mole of protein. Treatment of luciferase with [1-^{14}C]NEM in the same manner, followed by tryptic digestion and isolation of the radioactive peptides, resulted in a single ninhydrin-positive radioactive peptide referred to as LT-2.

The amino acid compositions of both LT-1 and LT-2 are shown in Table I. Since both appeared to be very nearly alike, partial acid hydrolysis was attempted on each followed by high-voltage paper electrophoresis of the digest. Identical patterns were obtained indicative of the fact that both peptides had the same amino acid sequence.

Amino Acid Sequence of Peptide LT-1. The results listed in Table I suggest that peptide LT-1 is a decapeptide. The mobility of this peptide at pH 3.7 and 6.5 indicated that it carried a balance of one net negative charge. In order to determine the sequence of amino acids from the amino terminal, peptide LT-1 was subjected to five successive Edman degradations and the composition of the residual peptides determined. The results obtained are summarized in Table II. These data permit us to conclude that the sequence of amino acids from the amino-terminal end of the peptide is at least Ser-CysSH-Glu-Gly-Asp, without assignment of amide linkages. Digestion of peptide LT-1 with carboxypeptidase A for 24 hr followed by amino acid analysis released no amino acids, indicative of a carboxyl-terminal lysine.

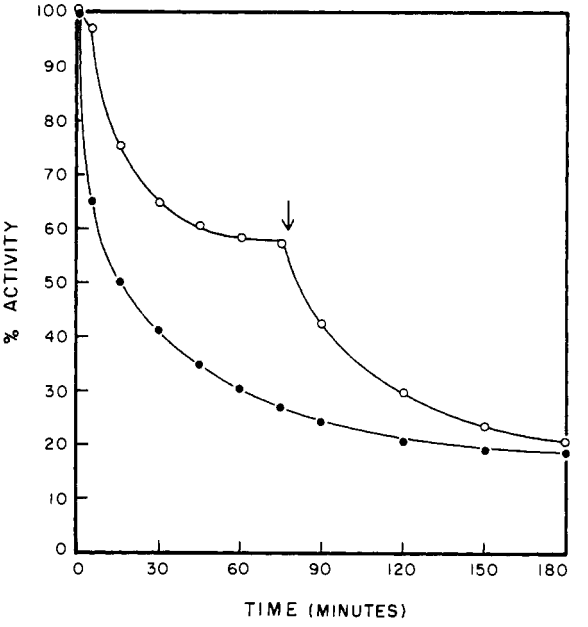

FIGURE 2: Inhibition of firefly luciferase by NEM. ●, addition of NEM at a 2:1 molar ratio. ○, addition of NEM at a 1:1 molar ratio. Arrow indicates the addition of a second mole of NEM.

TABLE II: Amino Acid Composition of Residual Peptides after Edman Degradation of LT-1.

	Degradation No.[b]					
Amino Acid[a]	0	1st	2nd	3rd	4th	5th
SSC	0.94	0.98	*0.05*	0.00	0.00	0.00
Aspartic acid	1.23	1.21	1.05	0.91	0.98	*0.59*
Serine	2.11	*1.00*	1.20	1.30	1.02	1.12
Glutamic acid	1.97	2.01	1.86	*0.84*	1.18	1.01
Glycine	2.13	2.05	2.03	2.00	*1.43*	1.30
Alanine	1.01	1.04	0.85	0.82	0.99	0.78

[a] Lysine was not determined during these reactions. [b] The amino acid removed in each cycle is italicized.

TABLE III: Edman Degradation of Peptides from Subtilisin Digests of LT-1.[a]

Amino Acids (Residues/ Mole)	Peptide								
	LT-S_1[b]		LT-S_2	LT-S_3			LT-S_4		
	Compn	1st Cycle	Compn	Compn	1st Cycle	2nd Cycle	Compn	1st Cycle	2nd Cycle
SSC	0.85	0.70							
Asp	0.97	0.85							
Ser	1.20	*0.40*		1.21	1.20	*0.40*	1.30	0.85	0.90
Glu	1.11	1.25		0.97	0.85	1.00	1.26	1.05	1.00
Gly	1.34	1.30		1.35	*0.15*	0.10	1.06	1.01	*0.35*
Ala	1.00	1.05	1.00				1.08	*0.34*	0.20
Lys							1.18	1.05	1.20

[a] The amino acid removed in each cycle is italicized. [b] Peptide was radioactive.

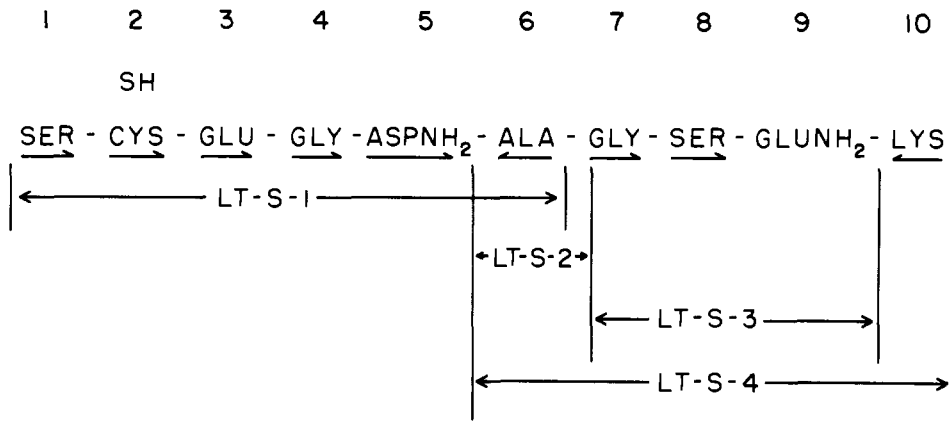

FIGURE 3: Amino acid sequence of LT-1. →, Edman degradation; ←, action of carboxypeptidase A.

Further degradation of LT-1 was carried out with subtilisin. High-voltage electrophoresis of the digest resulted in the separation of four main fractions, two neutral (LT-1-S_2 and LT-1-S_3), one acidic (LT-1-S_1), and one basic (LT-1-S_4). Only peptide LT-1-S_2 was radioactive. The composition of these peptides before and after Edman degradations is summarized in Table III.

Peptide LT-1-S_1 was acidic and had the following composition after acid hydrolysis: Asp,Ser,SSC,Glu, Gly,Ala. A single Edman degradation caused the loss of a serine residue indicating that this peptide must represent the amino-terminal sequence of peptide LT-1. Digestion with carboxypeptidase A for 12 hr resulted in the release of alanine and lesser amounts of asparagine. Since peptide LT-1-S_1 was acidic the sequence must have been Ser-CysSH-Glu-Gly-Asn-Ala.

Peptide LT-1-S_2 upon acid hydrolysis yielded only alanine. Because only one alanine residue was found in peptide LT-1 free alanine must have been split out during subtilisin digestion.

Peptide LT-1-S_3 was a neutral tripeptide which upon acid hydrolysis yielded equal amounts of glycine, serine, and glutamic acid. Two Edman degradations resulted in the loss of first glycine and then serine. The sequence of this peptide must then be Gly-Ser-Gln.

Peptide LT-1-S_4 was the only basic peptide isolated. Amino acid analysis gave the composition as follows: Ser,Glu,Gly,Ala,Lys. After two Edman degradations alanine and glycine had been split off in that order. These results, together with the basicity of the peptide, would suggest that it contains peptide LT-1-S_3 and what was believed to be peptide LT-1-S_2. The sequence of this peptide must be Ala-Gly-Ser-Gln-Lys. The total amino acid sequence of peptide LT-1 may now be written as shown in Figure 3.

Subunit Structure of Firefly Luciferase. The observation that only a single radioactive polypeptide could be isolated from digests of the labeled enzyme, where two would be expected, increased the possibility that the active form of the enzyme was composed of two identical subunits. To determine whether or not luciferase was composed of two or more monomers, sedimentation analysis was carried out by analytical centrifugation in buffered solutions and in unbuffered guanidine hydrochloride.

At concentrations <3 mg of protein/ml, firefly luciferase exhibits a loss in enzymatic activity and free SH content both of which are probably associated with disulfide formation. Furthermore, low- and high-speed equilibrium studies of such solutions yield plots with upward curvature, indicative of particle heterogeneity. For this reason all Yphantis studies were performed on solutions in which the SH residues had been alkylated with NEM. All low-speed equilibrium studies performed in 10% ammonium sulfate, pH 7.8, gave values of 89,000–91,000 for the molecular weight of luciferase. High-speed centrifugations in 0.1 M Tris, pH 7.0, resulted in molecular weights of from 92,000 to 95,000. In neither case was heterogeneity observed.

Preliminary sedimentation velocity studies on native luciferase yielded an $s_{20,w}$ of about 3.81. In 5 M guanidine hydrochloride, however, the boundary characteristic of the native enzyme is completely transformed into a much slower sedimenting component with an $s_{20,w}$ of 1.64. Sedimentation equilibrium of the alkylated enzyme in this dispersant again resulted in linear plots and a molecular weight of from 50,000 to 52,000.

In order to substantiate the centrifuge data which were indicative of a dimer, fingerprints of tryptic digests of oxidized luciferase were prepared. Amino acid analyses of the protein (DeLuca *et al.*, 1964) suggested that about 87–90 peptides should be obtained upon tryptic digestion if luciferase is composed of a single polypeptide chain. The number of ninhydrin-reactive peptides actually observed was between 42 and 45 or about one-half that expected on the basis of amino acid composition. Such results would be expected if luciferase is composed of two polypeptide subunits with identical amino acid sequence.

Amino-terminal analyses of the [^{3}HD]NP-labeled protein resulted in the isolation of a single DNP-amino acid after chromatography in the Blackburn and Lowther system (1951). The R_F of this component in-

dicated that it was DNP-serine. Quantitative measurements of the DNP-amino acid gave corrected values of 1.6–1.7 residues of DNP-serine/mole of luciferase. This, again, is in accord with the previous experiments indicating two identical subunits.

Discussion

The implication of SH groups at the active site of a variety of enzymes has been well documented (Perham and Harris, 1965; Gold and Segal, 1964; Li and Vallee, 1964; Wong and Liener, 1964). A unique property of several of these systems resides in the unusual reactivity of the SH group or groups intimately associated with enzyme catalysis. The most outstanding example of this occurs in yeast alcohol dehydrogenase (Harris *et al.*, 1963) where total inhibition occurs when only four of the 36 SH groups are alkylated. In the presence of substrate or NADH no inactivation occurs, suggesting a protection of the SH groups at the active site, or, alternatively, a conformational change whereby the previously exposed SH groups become buried.

In the present investigation amino acid analysis and partial acid hydrolysis of the radioactive peptides LT-1 and LT-2 proves, unambiguously, that the two SH groups covered by dehydroluciferyl adenylate and the reactive pair in the native enzyme are the same. Moreover, the isolation of a single radioactive decapeptide not only suggests a dimeric form of the native enzyme but also indicates that both SH groups contribute to the active site. This is further substantiated by the inhibition studies performed on the native enzyme with NEM where the addition of only 1 mole of NEM results in approximately 50% inactivation. Two interpretations which can be made from such data are that either 100% of all of the luciferase molecules have become damaged or 50% are totally inhibited and 50% are fully active. Kinetic studies with both native and 50% inhibited enzyme resulted in no observable change in the K_m for luciferin.[2] While this does not exclude the possibility of a monosubstituted molecule it is probable such a modified enzyme would have a different K_m for luciferin.

Although the sequence of the decapeptide isolated is not unusual it should be noted that, like glyceraldehyde 3-phosphate dehydrogenase and creatine phosphokinase, in which at least one free SH group is required, luciferase contains a particular asparagine residue three amino acids removed from the reactive SH group. When this is compared to yeast alcohol dehydrogenase, liver alcohol dehydrogenase, and lactic acid dehydrogenase in which aspartyl rather than an asparaginyl residue is found, the possibility of at least one structural analogy among SH enzymes becomes more plausible. However, until the investigation of many other SH-dependent enzymes has been undertaken, no clear rules may be firmly established. In the case of luciferase a comparison with the amino acid activating enzymes as suggested by DeLuca *et al.* (1964) would be of definite interest.

Ultracentrifugal analysis of native, catalytically active luciferase yields a molecular weight of 92,000. This value is slightly lower than that reported earlier by Green and McElroy (1956). Dissociation of the enzyme into two subunits of mol wt 52,000 occurs readily in 5 M guanidine hydrochloride, indicating that luciferase contains no disulfide bonds. That both subunits are alike is supported not only by the isolation of a single radioactive peptide where two would be expected but also by the identification of approximately one-half as many ninhydrin-positive peptides as theoretically possible on the basis of either nonidentical subunits or a single polypeptide chain. These results, together with the identification of two N-terminal serine residues, support the view that luciferase is composed of two identical monomeric units.

It is obvious that other amino acid residues must play an important part in the formation of the active site of luciferase. In the case of trypsin and chymotrypsin functional reagents have been prepared which specifically react with and thus implicate histidyl residues at the active site of each of these enzymes (Ong *et al.*, 1964; Shaw *et al.*, 1965). The preparation of such reagents was designed in order to obtain an inhibitor whose structure was quite similar to a substrate and contained a reactive functional group. Because natural luciferin has a structure ideally adaptable for the preparation of an alkylating reagent, analogs are currently being examined with the hope of extending knowledge of the active site of luciferase.

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