Role of Sulfhydryl Groups in Firefly Luciferase*

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The number of sulfhydryl groups of firefly luciferase was determined by spectrophotometric titration with *p*-mercuribenzoate in the presence and absence of a competitive inhibitor. Between six and seven sulfhydryls are titrated with *p*-mercuribenzoate in the native enzyme. In the presence of the inhibitor only four to five sulfhydryls will react with the *p*-mercuribenzoate. Four or five moles of *p*-mercuribenzoate can be reacted with the enzyme-inhibitor complex, and subsequent removal of the inhibitor results in recovery of 90% of the original enzymatic activity. Addition of 4 moles of *p*-mercuribenzoate to the enzyme in the absence of inhibitor results in complete loss of activity. The enzyme is also inhibited by dithiol reagents such as arsenite-2,3-dimercaptopropanol, $CdCl_2$, and γ-(*p*-arsenosophenyl)-*n*-butyric acid. The data show that four or five of the enzyme sulfhydryls have no effect on the catalytic activity, but the two sulfhydryl groups which are "covered" by the inhibitor are essential in some way for the enzymatic reactions leading to light emission. The amino acid composition of the protein was determined and spectrographic analysis showed the absence of any metal in stoichiometric amounts, thereby excluding the possibility of a metal cofactor.

Purified firefly luciferase (Green and McElroy, 1956), catalyzes the following reactions:

$$LH_2 + ATP + E \rightleftharpoons E\text{-}LH_2\text{-}AMP + P\text{-}P \quad (1)$$

$$E\text{-}LH_2\text{-}AMP + O_2 \longrightarrow \text{products} + \text{light} \quad (2)$$

The formation of enzyme-bound luciferyl-adenylate (E-LH_2-AMP) described in reaction (1) is analogous to the activation of fatty acids and amino acids and will be referred to as the *activating reaction*. The reaction of oxygen with the product of the activating step leads to light emission. The products of reaction (2) have not been definitely established, although dehydroluciferin (L) is formed during the reaction. In addition to reactions (1) and (2), luciferase will catalyze the formation of dehydroluciferyl-adenylate (L-AMP), equation (3), and in the presence of CoA luciferyl-CoA is formed as shown in reaction (4).

$$L + ATP + E \rightleftharpoons E\text{-}L\text{-}AMP + P\text{-}P \quad (3)$$

$$E\text{-}L\text{-}AMP + CoA \rightleftharpoons L\text{-}CoA + E + AMP \quad (4)$$

The properties of these reactions have been reported in detail (McElroy and Seliger 1963; Rhodes and McElroy, 1958).

The present study was undertaken to obtain information about the participation of functional groups of the enzyme in the catalysis.

It has been found that formation of enzyme dehydroluciferyl-adenylate complex masks at least two of the protein sulfhydryl groups, and inhibition by arsenite-BAL[1] and γ-(*p*-arsenosophenyl)-*n*-butyrate suggest these may be vicinal. Evidence is presented that the other sulfhydryl groups of the enzyme can be converted to the mercaptide derivative without impairing catalytic activity.

Experimental

Materials.—Five-times-crystallized firefly luciferase was prepared as described by Green and McElroy (1956). The enzyme was stored as 0° as a concentrated solution, 10–20 mg protein/ml, in 10% ammonium sulfate, 10^{-3} M EDTA, *p*H 7.5. This stock solution was diluted just prior to use.

Crystalline *D*(−)luciferin and dehydroluciferin were synthesized according to White *et al.* (1961). The concentration of the luciferin was determined by measuring the absorbance at neutral *p*H at 327 mμ, or for dehydroluciferin at 347 mμ.

Luciferyl-adenylate was synthesized essentially according to the procedure of Rhodes and McElroy (1958). Some preparations were found to contain a compound inhibitory to the enzyme which could be removed by more extensive extraction with ethyl acetate at *p*H 2. The concentration of luciferyl-adenylate was measured by the increase in fluorescence (activation 327 mμ, emission 530 mμ) after alkaline hydrolysis. The fluorescence was then compared with the fluorescence of luciferin of known concentrations.

PMB was obtained from Sigma Chemical Co. and recrystallized before use. PMB solutions were prepared in 0.1 M Tris, *p*H 7.1, to an approximate concentration of 1×10^{-3} M. The PMB concentration was determined spectrophotometrically at 232 mμ using an A_M of 1.69×10^4 (Boyer, 1954).

BAL was obtained from the Aldrich Chemical Co. It was stored under nitrogen and fresh solutions were made daily and used immediately. γ-(*p*-Arsenosophenyl)-*n*-butyric acid was generously supplied by Dr. Leslie Hellerman. All other chemicals were of reagent grade and were used as obtained.

Methods.—Protein concentration was measured by absorbance at 278 mμ. One mg of luciferase/ml in a 1-cm cell gives an optical density of 0.75. A molecular weight of 100,000 was used for calculations of enzyme concentration (Green and McElroy, 1956).

Enzyme Assays.—Enzymatic activity was assayed by measuring the intensity of the initial flash of light emitted as described by McElroy and Seliger (1961). The combined activating and light-emitting reactions were measured by adding enzyme to a solution of 0.025 M glycylglycine buffer at *p*H 7.5, containing 0.004 M $MgCl_2$ and 3×10^{-5} M luciferin in a final volume of 2.5 ml. The reaction was initiated by rapidly injecting 0.2 ml of 0.02 M ATP, *p*H 7.5, with a hypodermic syringe. The maximum light intensity occurs within 2 seconds and the height of the peak was taken as a measure of initial velocity and expressed in arbitrary inten-

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[1] Abbreviations used in this paper: BAL, 2,3-dimercaptopropanol; PMB, *p*-mercuribenzoate.

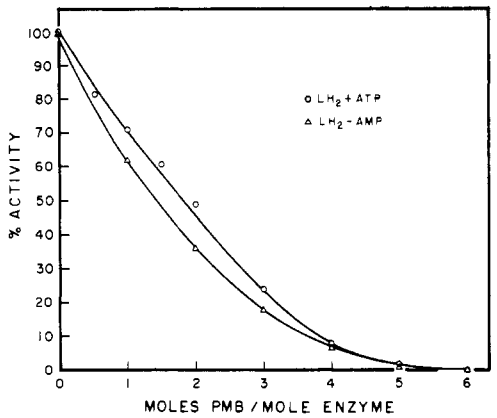

FIG. 1.—Loss of enzymatic activity as a function of —SH groups reacted with PMB. O, loss of activating and light-emitting reactions; Δ, loss of light-emitting reaction. Activity is expressed as the per cent of a control sample with no PMB added. Assays are as described under Methods.

sity units. Since some variability in light intensity arises as a result of the mixing of ATP, all assays were carried out in triplicate and the average value was reported.

The light-emitting step, reaction (2), was measured in an analogous manner, except that the reaction was initiated by the injection of synthetic luciferyl-adenylate into a tube containing buffer and enzyme. The reported values in all cases are averages of four or more assays.

Both assays were proportional to enzyme concentration over the range studied. Linearity was established with each new preparation of luciferase, and with every preparation of luciferyl-adenylate. The specific activity of the enzyme preparations used varied from 90,000 to 125,000 intensity units/mg protein.

The number of –SH groups was determined using PMB according to a procedure of Benesch and Benesch (1962) based on the work of Boyer (1954). In a typical experiment 0.07 ml of a luciferase solution was diluted to 1.0 ml with the appropriate buffer in a 1.0-ml silica cuvet. The contents was mixed and the protein concentration was determined by measuring the absorbance at 278 mμ. PMB (approximately 10^{-3} M) was added in increments of 0.006–0.010 ml, and 30–45 minutes was allowed for the reaction to occur. Optical density at 250 mμ was measured after each addition of PMB. During the titration it was necessary to keep the enzyme-PMB mixture cold, as any warming resulted in immediate precipitation of the protein. The results of such a titration are plotted as shown in Figure 2, and the volume of PMB necessary to titrate the exposed –SH groups was determined from the break in the curve. The absorption measurements were made with a Zeiss PMQ 11 spectrophotometer.

Amino acid analysis. Extensive dialysis of luciferase against distilled water was followed by centrifugation and washing of the insoluble protein to remove all ammonium sulfate. Duplicate samples of the enzyme were subjected to hydrolysis *in vacuo* in 6 N HCl for 24, 48, and 72 hours. The hydrolysates were analyzed on the Beckman/Spinco automatic amino acid analyzer according to Spackman *et al.* (1958). Half-cystine was determined as cysteic acid in hydrolysates of the protein after performic acid oxidation by the method of Hirs (1956). Protein concentration was determined by the micro-Kjeldahl procedure.

TABLE I

LOSS OF ENZYMATIC ACTIVITY AS A FUNCTION OF TIME OF INCUBATION WITH PMB[a]

Moles PMB/Mole Enzyme	Control (%)			
	Activating and Light-emitting (Luciferin + ATP)		Light-emitting (luciferyl-adenylate)	
	30 min	40 hr	30 min	40 hr
0.5	82	79	74	72
1.0	75	67	64	63
1.5	63	45	44	45
2.0	51	37	39	40

[a] The samples contained approximately 3×10^{-6} M luciferase in 0.025 M glycylglycine, *p*H 7.5. PMB was added as shown and the samples were assayed as described in the methods 30 minutes after the addition of the PMB. They were stored at 0° for 40 hours and reassayed. The activity is expressed as per cent of a control sample which was treated identically except that no PMB was added.

RESULTS

Titration of Sulfhydryl Groups of Luciferase.—The –SH content of luciferase was determined with PMB as described under Methods. For three different preparations of the enzyme, between 6 and 7 –SH groups were found to react with PMB. Figure 1 shows the loss of enzymatic activity as a function of the number of –SH groups titrated. The light-emitting reaction is inhibited to a somewhat greater extent than the activating reaction. This difference in loss of activity is dependent upon the length of time the enzyme is allowed to incubate with PMB prior to assay. The data in Table I show that after 30 minutes of incubation with PMB there is a real difference in the activities of the two reactions. If these samples are assayed after 40 hours, the per cent loss of activity for both reactions is the same.

During the first 30 minutes after the addition of PMB the loss of enzyme activity follows closely the absorbancy changes accompanying mercaptide formation. The first 3 moles of PMB added per mole of enzyme react completely within 15 minutes at 0° while the next three require from 30 to 45 minutes for complete reaction. The subsequent loss in enzyme activity as shown in Table I occurs very slowly and without further change in absorbance at 250 mμ. Both the initial and the gradual loss of activity are reversed almost completely by the addition of excess cysteine.

Effect of dehydroluciferyl-adenylate on Reactive Sulfhydryls.—Dehydroluciferin will react in the presence of luciferase, Mg^{2+}, and ATP to form enzyme dehydroluciferyl-adenylate, which is a potent competitive inhibitor of the natural substrate, luciferyl-adenylate. Dehydroluciferyl-adenylate is very tightly bound to the enzyme (Rhodes and McElroy, 1958). Several PMB titrations were carried out in the presence of dehydroluciferyl-adenylate to see if any of the protein –SH groups were prevented from reacting with PMB when dehydroluciferyl-adenylate was on the enzyme. These data are given in Table II. In each experiment two titrations were carried out simultaneously; one cuvet contained only enzyme and buffer and the other contained in addition ATP, Mg^{2+}, and a 6 × molar excess of dehydroluciferin. It can be seen that, in the presence of dehydroluciferyl-adenylate, two or three fewer –SH groups react with PMB. Figure 2 is an example of the titration curves obtained in such an experiment. Several titrations were carried out in 6 M urea and in all cases six –SH groups per mole of protein were found.

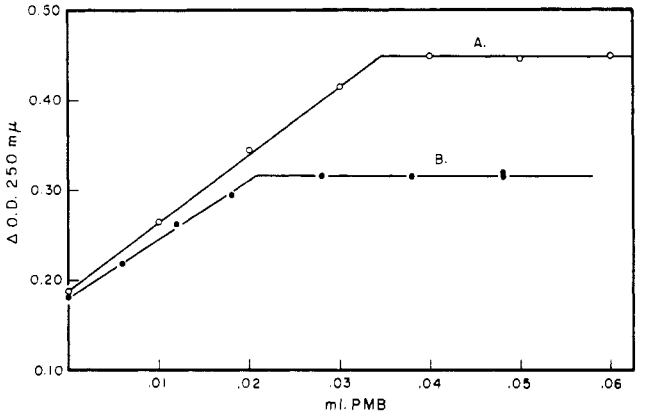

FIG. 2.—PMB titration curve of the —SH groups of luciferase in the presence and absence of L-AMP. Curve A, 5.72 mμmoles luciferase in 0.025 M Tris buffer, pH 7.1. Curve B, 5.72 mμmoles luciferase, 32 mμmoles L, 45 mμmoles ATP, and 5 μmoles $MgSO_4$. Optical density changes were read against a blank of the same composition minus the protein. PMB concentration was 1.14×10^{-3} M.

In subsequent experiments, advantage was taken of the ability of CoA to react with enzyme dehydroluciferyl-adenylate to form dehydroluciferyl-CoA and free enzyme. The enzyme was first treated with dehydroluciferin, ATP, and Mg^{2+} as described in Table III, then 4 or 5 moles of PMB per mole of enzyme were added and allowed to react 60 minutes at 0°. Essentially all of the enzyme activity was lost either with dehydroluciferyl-adenylate, dehydroluciferyl-adenylate and PMB, or PMB alone after this time of incubation. Approximately a 10 × molar excess of CoA was added and the enzyme was assayed by flash height with luciferin and ATP. A similar sample of the enzyme was reacted with the same amount of PMB in the absence of dehydroluciferyl-adenylate. This sample served as a control, since the CoA would be expected to remove some of the PMB by nonspecific reaction as a sulfhydryl compound. The data in Table III show that the enzyme which was protected by dehydroluciferyl-adenylate (sample 2) recovers almost as much activity as the sample in which no PMB was added (sample 1). The unprotected enzyme showed much less activity restored upon the addition of CoA.

In experiment II, Table III, sample 3 again shows further loss of activity upon prolonged incubation with PMB, even in the presence of CoA. This was not observed when the enzyme had been preincubated with dehydroluciferyl-adenylate (sample 2). All these samples showed complete recovery of activity upon the addition of excess cysteine.

TABLE II
EFFECT OF DEHYDROLUCIFERYL-ADENYLATE ON THE NUMBER OF REACTIVE—SH GROUPS OF LUCIFERASE[a]

Number of Reactive—SH/Mole of Luciferase		
Without Dehydroluciferyl-adenylate	With Dehydroluciferyl-adenylate	Difference
6.1	4.1	2.0
7.1	5.0	2.1
6.7	4.1	2.6
6.8	3.9	2.9
7.0	4.0	3.0
6.0	4.0	2.0

[a] Stock luciferase (0.07 ml) was diluted to 1.0 ml with 0.025 M Tris, pH 7.1. Where dehydroluciferyl-adenylate was present, 3.2 mμmoles of dehydroluciferin, 4.5 mμmoles ATP, and 5 μmoles of $MgSO_4$ were added. The enzyme was allowed to react for 30 minutes at 0° with the dehydroluciferin, ATP, and Mg^{2+} before the titration was started. Titrations were carried out as described under Methods.

TABLE III
PROTECTION OF LUCIFERASE FROM PMB-INACTIVATION BY DEHYDROLUCIFERYL-ADENYLATE[a]

Additions	Original Activity (%)	Original Activity after Addition of CoA (4× excess) (%)	
Experiment I			
1. Enzyme, dehydroluciferyl-adenylate	5, 6	100, 100	
2. Enzyme, dehydroluciferyl-adenylate, PMB (4× excess)	2, 3	90, 90	
3. Enzyme, PMB (4× excess)	1, 0	24, 28	
Experiment II			
		2 hr	4.5 hr
1. Enzyme, dehydroluciferyl-adenylate	5	100	100
2. Enzyme, dehydroluciferyl-adenylate, PMB (5× excess)	2	74	84
3. Enzyme, PMB (4× excess)	1	23	13

[a] All samples contained 3 mμmoles of luciferase; where indicated, 15 mμmoles dehydroluciferin, 5 mμmoles ATP, and 1 μmole $MgSO_4$ were added to the enzyme prior to PMB. After the addition of PMB the reaction was allowed to proceed for 60 minutes at 0° and duplicate aliquots were removed and assayed. Coenzyme A was then added and assays were performed at the times indicated. In expt I, PMB was in a 4 × molar excess and CoA was 4 × excess. In expt II, PMB was in 5 × molar excess of luciferase and CoA was 10 × molar excess. Assay was with luciferin and ATP as described under Methods.

Effect of Cd^{2+} and Arsenite-BAL.—The previous experiments have shown that dehydroluciferyl-adenylate "masked" two or three of the –SH groups of luciferase. It was of interest to see if dithiol inhibitors had any effect on the light emission. Table IV gives the results of these experiments. Cd^{2+} and arsenite in the presence of BAL inhibit the enzyme. The amount of inhibition with a given concentration of arsenite-BAL was greater with longer times of exposure to the enzyme. Arsenite in the presence of an equal amount of cysteine inhibits to a lesser extent, while arsenite alone is only slightly inhibitory. A 20 × molar excess of cysteine over the arsenite will partially reverse the inhibition of arsenite-BAL, while a 4 × molar excess of BAL completely reverses the inhibition. If the arsenite-BAL is incubated with the enzyme in the presence of 1×10^{-2} M $MgCl_2$, the inhibition is not as great. A similar effect has been observed by Fluharty and Sanadi (1961) with mitochondrial systems. It should be noted that the concentration of arsenite-BAL in the final assay mixture is much lower than that of the preincubation mix, since an aliquot is diluted 25 × for the final assay.

Experiment 4 shows that the enzyme is also sensitive to low concentrations of $CdCl_2$. $ZnCl_2$ inhibits to a similar degree at 1×10^{-4} M. If the $CdCl_2$ is added in the presence of substrate, the inhibition observed is considerably less. This effect of $CdCl_2$ can be completely reversed by the addition of cysteine or EDTA.

TABLE IV
EFFECT OF DITHIOL REAGENTS ON LUCIFERASE

Expt[a]	Additions (final molar concn)	Preincubation Time (min)	Inhibition (%)
1	Arsenite, 1×10^{-3} M	5	4
		30	9
	BAL, 1.3×10^{-3} M	5	0
		30	0
	Arsenite, 1×10^{-3} M	5	50
	BAL, 1.3×10^{-3} M	30	83
2	Cysteine, 1×10^{-3} M	30	0
	Arsenite, 1×10^{-3} M	30	20
	Cysteine, 1×10^{-3} M Arsenite, 1×10^{-3} M	30	40
	BAL, 6×10^{-3} M Arsenite, 1×10^{-3} M	30	6
3	γ-(*p*-Arsenosophenyl)-*n*-butyrate		
	1×10^{-4} M	50	98
	5×10^{-5} M	50	92
	5×10^{-6} M	50	89
4	$CdCl_2$, 5×10^{-6} M	3	64
	1×10^{-5} M	3	72
	5×10^{-5} M	3	88
	1×10^{-4} M	3	92

[a] Experiments 1, 2, and 3: the final mix was approximately 2.6×10^{-6} M enzyme; inhibitor concentrations were as specified. Final volume 0.6 ml, 0.025 M glycylglycine, pH 7.5. Preincubation time is the time the enzyme and inhibitor were incubated at 0° prior to assay. Aliquots of each sample were removed and assayed as described previously. Experiment 4: $CdCl_2$ was added to the assay mix without preincubation with the enzyme. An aliquot of enzyme was added and 3 minutes later the substrate and Mg^{2+} were added and the assay was done immediately.

Inhibition by γ-(*p*-arsenosophenyl)-*n*-butyrate is almost complete at very low concentrations if it is allowed to incubate with the enzyme for 50–60 minutes prior to assay.

The emission spectra of the luciferase reaction was measured in the presence of arsenite-BAL, PMB, and γ-(*p*-arsenosophenyl)-*n*-butyrate under conditions where the enzyme was inhibited approximately 50%, and no change in the color of the emitted light was observed. However, $CdCl_2$ at 1×10^{-3} M caused a shift in the light about 500 A to the red. This shift was partially reversed upon the addition of excess cysteine.

Amino Acid Composition and Metal Content of Luciferase.—The amino acid composition of luciferase is given in Table V. Performic acid oxidation on three different samples of luciferase show a maximum value of 10 half-cystine residues per mole of enzyme, indicating that not more than two disulfides can be present, if a value of 6 is assumed for free –SH groups. The reason for the variability among these samples could be owing to incomplete oxidation or could be a reflection of a small amount of contaminating protein.

Luciferase, as an oxidative enzyme, is somewhat unusual since it has no known cofactors necessary for catalytic activity. The possibility that a tightly bound metal might participate in the catalysis had not been examined. A sample of purified luciferase was analyzed for metal content by spectrographic procedures[2] and found to contain no metal in significant quantities. Co, Zn, Mn, Cd, Mo, Pb, Fe, Ni, and Cr, were undetectable. Mg was less than 0.3 mole/mole protein; Al

[2] We would like to thank Dr. B. L. Vallee for performing the metal analyses.

TABLE V
AMINO ACID COMPOSITION OF LUCIFERASE

	μMoles/mg Protein[a]
Lysine	0.541
Histidine	0.232
Ammonia	0.888
Arginine	0.336
Aspartic acid	0.784
Threonine	0.450
Serine	0.460
Glutamic acid	0.885
Proline	0.467
Glycine	0.654
Alanine	0.607
Valine	0.655
Methionine	0.248
Isoleucine	0.609
Leucine	0.820
Tyrosine	0.335
Phenylalanine	0.547
Cysteic acid[b]	0.104, 0.102
	0.087, 0.092
	0.068, 0.069

[a] Average values from triplicate determinations (22-hr hydrolysates), with the exception of serine and threonine, which were corrected to 0-time hydrolysis. [b] Values are given from duplicate samples determined on three different enzyme preparations. Protein concentration is based on total nitrogen recovered from the column.

was less than 0.2 mole/mole protein; and Sr, Ca, and Ba were all less than 0.01 mole/mole protein. Lack of stoichiometric amounts of these metals is good evidence that they do not participate in the catalytic activity of luciferase.

DISCUSSION

The masking of two or three –SH groups of luciferase by the competitive inhibitor dehydroluciferyl-adenylate together with the almost complete recovery of activity from the protected enzyme (Table III) gives strong support for the involvement of these –SH groups either in the catalytic activity of the enzyme or in the maintenance of a specific conformation at the active site. As in all such studies, this kind of evidence cannot be taken as proof for the direct participation of the –SH groups in catalysis; however, it can be stated with certainty that the four or five –SH not covered by the dehydroluciferyl-adenylate are not essential in any way to the activity of the enzyme, since 90% of the activity can be recovered with these –SH groups in the form of the mercaptide derivative of PMB.

It should be pointed out that the data in Figure 1 and in Table I, which show the light-emitting activity is more sensitive to lower concentrations of PMB than the activating reaction, do not mean that the light-emitting step is the rate-limiting reaction. The assay is a measure of the initial reaction rate and the data are expressed as a per cent of a control sample containing identical amounts of enzyme and the appropriate substrate in the absence of PMB. Since large amounts of luciferyl-adenylate were not available, these assays were carried out at concentrations below saturation of the enzyme, and the velocity measured was not V_{max}. However, the assay using luciferin and ATP was carried out with excess of both substrates and this velocity was proportional to V_{max}.

The initial loss of activity of the light-emitting reaction as a function of PMB concentration (Fig. 1) follows closely the calculated values assuming equal reactivity of all sulfhydryls and that mercaptide formation of one of the two essential groups results in complete loss of

enzymatic activity. The further loss of activity after 40 hours with a given concentration of PMB (Table I) could be explained by a slow structural change occurring or possibly the rearrangement of a PMB from one –SH to another. Any such change must be rapidly and completely reversed by cysteine, since even after 40 hours full activity can be recovered in the presence of excess cysteine.

Several other systems have been reported where BAL is required with arsenite for inhibition (Sekuzu *et al.*, 1963; Fluharty and Sanadi, 1961), and it has been suggested by Fluharty and Sanadi (1961) that the role of BAL is to transport the arsenite to the dithiol groups of the protein. The most convincing evidence for dithiol involvement in luciferase activity is the complete reversal of the arsenite inhibition by BAL, whereas cysteine at higher concentrations only partially reverses the inhibition. It is possible that arsenite may be reacting both with a dithiol and a single –SH group at these concentrations.

Jakoby (1958) has reported inhibition of several aldehyde oxidases by arsenite in the presence of monothiols, and has suggested that the thiol was acting to reduce a protein disulfide which could then react with the arsenite. This would seem not to be the explanation in the case of luciferase since γ-(*p*-arsenosophenyl)-*n*-butyrate inhibits the enzyme in the absence of any added thiol. If this compound is acting by binding a vicinal dithiol, this would suggest that the dithiol is present as such in the native enzyme and the masking of two –SH groups by the dehydroluciferyl-adenylate was not the result of an induced structural change by the inhibitor.

It has been shown by Reiss (1958) that, *in vitro*, reduced lipoic acid reacts rapidly and stoichiometrically with the arsenoso reagent at *p*H 7.4. Inhibition of luciferase with this reagent requires from 30 to 50 minutes, which suggests that the dithiol is perhaps "buried." This possibility is substantiated by the fact that PMB does not react instantaneously, as well as by the observation that the enzyme even in dilute solutions does not lose activity as rapidly as would be expected if the –SH groups were easily autoxidized.

It is interesting to note that Hastings and Gibson (1963) have reported that one of the intermediates in the bacterial luciferase is also arsenite-sensitive, suggesting the possibility that –SH groups may be important in the oxidative reaction that leads to the creation of an excited state.

The effect of Cd^{2+} on luciferase appears to be different from that of the arsenite compounds since it causes a shift in the emission spectrum of the enzyme, similar to that observed with Zn^{2+}.

It would be of interest to see if other enzymes which catalyze formation of acyl adenylates, such as the acetate-activating and amino acid–activating enzymes, show similar –SH involvement. It is possible that the dithiol may in some way be responsible for the characteristically tight binding of the acyl adenylates to the enzymes. Further experiments are in progress to clarify the function of these groups in the catalytic process.

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